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HRT1, *HRT2*, and *HRT3*: A New Subclass of bHLH Transcription Factors Marking Specific Cardiac, Somitic, and Pharyngeal Arch Segments

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Members of the Hairy/Enhancer of Split family of basic helix-loop-helix (bHLH) transcription factors are regulated by the Notch signaling pathway in vertebrate and *Drosophila* embryos and control cell fates and establishment of sharp boundaries of gene expression. Here, we describe a new subclass of bHLH proteins, HRT1 (Hairy-related transcription factor 1), HRT2, and HRT3, that share high homology with the Hairy family of proteins yet have characteristics that are distinct from those of Hairy and other bHLH proteins. Each *HRT* gene was expressed in distinct cell types within numerous organs, particularly in those patterned along the anterior–posterior axis. *HRT1* and *HRT2* were expressed in atrial and ventricular precursors, respectively, and were also expressed in the cardiac outflow tract and aortic arch arteries. *HRT1* and *HRT2* transcripts were also detected in precursors of the pharyngeal arches and subsequently in the pharyngeal clefts. Within somitic precursors, *HRT1* and *HRT3* exhibited dynamic expression in the presomitic mesoderm, mirroring the expression of other components of Notch–Delta signaling pathways. The *HRT* genes were expressed in other sites of epithelial–mesenchymal interactions, including the developing kidneys, brain, limb buds, and vasculature. The unique and complementary expression patterns of this novel subfamily of bHLH proteins suggest a previously unrecognized role for Hairy-related pathways in segmental patterning of the heart and pharyngeal arches, among other organs.

INTRODUCTION

Patterning of organisms along the anterior–posterior (AP) axis proceeds in a segmental fashion whereby discrete embryonic domains are established concomitant with cell fate decisions. The sequential appearance of rhombomeres, somites, and pharyngeal arches represents the earliest morphologic evidence of body segmentation and involves cascades of signaling and transcriptional events (Krumlauf, 1993). Homeodomain-containing proteins and members of the basic helix-loop-helix (bHLH) family of transcription factors are often involved in such regulatory pathways and control embryonic patterning along multiple axes.

Individual organs are also patterned based on the positions of cells relative to embryonic axes. The heart is the first organ to form in mammalian embryos and is intricately patterned along all three axes. Cardiac mesodermal cells migrate from the primitive streak and become arranged in a crescent fashion in the anterior half of the embryo (Yutzey and Bader, 1995). Cells originating from the more anterior portion of the primitive streak, migrate to the anterior part of the cardiac crescent (DeHaan, 1965). The cardiac crescent develops into a midline straight heart tube that is patterned along the AP axis to give rise to the cardiac outflow tract, right ventricle, left ventricle, atria, and inflow tract (reviewed in Olson and Srivastava, 1996). Subsequent looping of the heart tube in the rightward direction converts the initial AP polarity into a left–right cardiac asymmetry (Levin, 1997).

Developmental cardiac defects in humans most often affect discrete regions or chambers of the heart, suggesting that distinct molecular pathways regulate individual segments of the primitive heart tube (Srivastava, 1999). Such a model is supported by the identification of chamber-specific *cis* elements in the regulatory regions of numerous

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genes, including *myosin light chain 2V* (*MLC2V*) (Ross *et al.*, 1996), *myosin light chain 1F* (Kelly *et al.*, 1995), *desmin* (Kuisk *et al.*, 1996), and *SM22* (Li *et al.*, 1996). After cardiac looping, many other proteins that were previously expressed homogeneously segregate specifically to the atrial or ventricular chambers (Lyons *et al.*, 1994). Although numerous cardiac transcription factors have been identified, the *trans*-acting factors that interact with chamber-specific *cis* elements and confer chamber specificity have not been identified.

Members of the MEF2 (Lin *et al.*, 1997), GATA (Molkentin *et al.*, 1997; Kuo *et al.*, 1997), and Nkx (Harvey, 1996) families of transcription factors are important in regulating cardiac development, but are expressed uniformly in all segments of the developing heart. In contrast, the bHLH proteins dHAND (Srivastava *et al.*, 1995) and eHAND (Cserjesi *et al.*, 1995) are expressed in a complementary fashion in the right and left ventricles, respectively (Srivastava *et al.*, 1997). Deletion of the *dHAND* gene in mice results in specific hypoplasia of the right ventricle, indicating that dHAND functions in a chamber-specific manner (Srivastava *et al.*, 1997). Recently, the Irx4 homeodomain protein was shown to be expressed in the ventricular but not atrial precursors of chick embryos (Bao *et al.*, 1999). Ectopic expression of Irx4 in the atria resulted in expression of a ventricle-specific myosin heavy chain, suggesting that Irx4 may be involved in establishing ventricle-specific gene transcription (Bao *et al.*, 1999).

Despite the recent identification of segment-specific cardiac transcription factors, the molecular basis for establishment of discrete boundaries of gene expression during cardiogenesis remains unknown. Studies in other organisms provide a framework for considering how sharp boundaries of cell fate are established during embryogenesis. In *Drosophila*, interaction of the Delta and Serrate ligands with Notch receptors on neighboring cells initiates feedback loops resulting in the establishment of regional differences in gene expression (Greenwald, 1998). One of the primary targets of Notch signaling is the family of Hairy-related bHLH transcription factors (Wettstein *et al.*, 1997; Takke *et al.*, 1999). Hairy proteins are thought to negatively regulate other bHLH proteins while establishing discrete boundaries of expression in somitic and neurogenic precursors. No Hairy-related bHLH protein has been shown to play a role in the heart or pharyngeal arches.

In an effort to identify novel cardiac bHLH proteins, we searched DNA sequence databases for novel bHLH proteins with homology to the bHLH regions of dHAND and eHAND. Here, we describe a new subfamily of three bHLH proteins that we refer to as HRT1 (Hairy-related transcription factor 1), HRT2, and HRT3. The HRT proteins share extensive homology with the bHLH regions of Hairy and HES but are divergent in several critical residues characteristic of the Hairy family. Their expression is dynamic during embryogenesis and is observed in specific and complementary segments of the developing straight heart tube and in the somites and pharyngeal arches. *HRT1* and *HRT2* are expressed in atrial and ventricular precursors, respectively, while *HRT3* transcripts are detected in postnatal hearts. Within the somites and pharyngeal arches, *HRT* transcripts were detected in the precursor cells of both structures before becoming regionally restricted along the AP axis. These data provide some of the earliest markers for segmentation of heart, somite, and pharyngeal arch precursors and suggest a role for HRT proteins in segmental patterning of tissues along the AP axis.

METHODS

cDNA Cloning of HRT Genes

The DNA clones in the Berkeley *Drosophila* Genome Project database were translated into six putative open-reading frames and compared with mouse dHAND and eHAND to find novel bHLH sequences using the tblastn program (Zheng *et al.*, 1998). One of the bHLH-like sequences in the genomic DNA clone DS06886 did not have corresponding *Drosophila* or mammalian genes in GenBank and was used to search the mouse expressed-sequence tag (EST) database using tblastn. A 369-bp cDNA fragment in a mouse EST (Accession No. AI181098) similar to the *Drosophila* sequence was amplified by RT-PCR using mouse whole-embryo RNA (upper primer, 5′-TCGCCACCATGAAGAGAG-3′; lower primer, 5′-CCATAGCCAGGGCGTGCG-3′). A mouse embryonic day 10.0 (E10.0) heart cDNA library (Stratagene) was screened using the ^{32}P-labeled mouse cDNA fragment in hybridization buffer composed of 30% formamide, 6× SSPE, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA, followed by two washes with 2× SSC, 0.1% SDS at room temperature. Positive bacteriophage clones were purified and cDNA was excised into pBluescript plasmids. DNA sequencing was performed on both strands by the cycle sequencing method.

Northern Blot Analysis

*Hin*cII, *Bsr*GI/*Sac*II, or *Apa*I/*Pst*I fragments of HRT1, HRT2, or HRT3, respectively, were used to prepare ^{32}P-labeled DNA probes that contained the open reading frame of each. Mouse adult tissue poly(A)$^+$ RNA blots (Clontech) were normalized using β-actin as control. Membranes were prehybridized and hybridized in RapidHyb buffer (Amersham) at 65°C and washed serially, with the final wash in 0.1× SSC, 0.1% SDS at 70°C. Autoradiography was performed at −80°C for 10 h with an intensifying screen.

In Situ Hybridization

Whole-mount *in situ* hybridization was performed by a modification of our previous methods (Yamagishi *et al.*, 1999). Digoxygenin-labeled RNA probes corresponding to the protein-coding regions of HRT1, -2, and -3 were prepared by *in vitro* transcription. Mouse embryos from E8.0 to E10.5 were isolated and the pericardium was removed. Embryos were then fixed in 4% paraformaldehyde for 4 to 8 h at 4°C. Embryos were treated with proteinase K (20 μg/ml) for 2 to 15 min at room temperature depending on their stage of development and fixed in 4% paraformaldehyde/0.2% glutaraldehyde for 10 min at room temperature. Prehybridization was performed at 61°C for 3 h in solution containing 50% formamide, 4× SSC, 1× Denhardt's

solution, 500 μg/ml herring sperm DNA, 250 μg/ml yeast tRNA, and 10% dextran sulfate; hybridization was performed with RNA probes in the same solution for 24 h. After a series of washes, embryos were treated in 2% blocking solution (Roche) for 1 h and incubated with anti-digoxygenin antibody (Roche) for 1 h at room temperature. Color reaction was performed at room temperature in substrate color reaction mixture (Roche) for 18–36 h and terminated by fixing embryos in 4% paraformaldehyde, 0.2% glutaraldehyde.

Radioactive section *in situ* hybridization was performed on paraffin-embedded sections of E10.5 and E15.5 embryos using ^{35}S-radiolabeled RNA probes for *HRT1, -2,* and *-3,* as previously described (Lu *et al.,* 1998).

Breeding of Mice and Genotyping of Embryos

Mice heterozygous for *Nkx2.5* or *dHAND* mutations were generated as previously described (Lyons *et al.,* 1995; Srivastava *et al.,* 1997). Intercrosses of *Nkx2.5* heterozygous mice in the 129SVEV/C57BL6 background were performed to obtain E9.5 homozygous-null embryos. Mice heterozygous for the *dHAND* mutation in the 129SV/C57BL6 background were similarly bred to obtain E9.5 homozygous-null embryos. Isolation of yolk sac DNA from embryos and genotyping of *Nkx2.5* or *dHAND* mutants by Southern blot analysis or PCR were performed as previously described (Lyons *et al.,* 1995; Srivastava *et al.,* 1997).

RESULTS

HRT1–3 Comprise a New Subfamily of bHLH Proteins

Because the structure and function of transcription factors are often conserved between vertebrates and flies, the *Drosophila* genome database affords a possibility of finding ancestral genes of mammalian transcription factors. We searched for gene products sharing homology with the bHLH proteins dHAND and eHAND in the *Drosophila* genome database. Numerous sequences sharing homology within the bHLH region were identified and will be described elsewhere. One of the *Drosophila* genomic sequences encoded a putative bHLH domain that shared approximately 35% amino acid identity with the mouse HAND bHLH domains but had no corresponding *Drosophila* genes or mammalian homologues reported in GenBank. This novel *Drosophila* sequence shared higher homology with the bHLH domain of *Drosophila Hairy* but was clearly distinct.

In order to identify a mouse counterpart of this bHLH sequence, we screened the EST database and found several overlapping mouse EST sequences that shared up to 84% predicted amino acid identity with the novel *Drosophila* bHLH domain. A PCR fragment corresponding to this mouse sequence was used to screen a mouse embryonic heart cDNA library to obtain a full-length cDNA clone. Because bHLH proteins often exist in small, closely related subfamilies, low-stringency screening conditions were used to identify other potential family members simultaneously. Sequencing of 17 independent cDNA clones from this screen allowed segregation of the clones into three distinct categories, each encoding a novel bHLH protein. We refer to these gene products as HRT1, HRT2, and HRT3.

Sequence comparison of HRT1–3 revealed that each encoded a protein containing a bHLH domain toward the amino-terminus (Fig. 1A). HRT2 and HRT3 shared 57 and 45% homology with HRT1 over the entire protein, but displayed regions of increased homology at the amino- and carboxy-termini (Fig. 1A). The highest region of homology was observed in the bHLH domain, where HRT2 and HRT3 shared 96 and 91% homology with HRT1, respectively (Figs. 1A and 1D). Comparison of the bHLH domain of HRT1–3 with the bHLH domain of other known bHLH proteins revealed the presence of critical residues typical of bHLH proteins (Fig. 1B). Only two amino acid mismatches, in identical residues for each HRT protein, were present compared to a predicted model of bHLH factors (Atchley *et al.,* 1999). The basic regions, or DNA-binding domains, of HRT1–3 were nearly identical, suggesting that HRT1–3 may interact with common promoter/enhancer DNA sequences. The HRT proteins had closest homology to a family of transcriptional repressors, including Hairy and HES-1 (Figs. 1B and 1C). They typically contain a conserved region adjacent to the bHLH region, known as an orange domain, that confers functional specificity among Hairy family proteins (Dawson *et al.,* 1995). The three HRT proteins are highly conserved in a region just C-terminal to the bHLH region and share significant similarity in this stretch with the orange domain of Hairy-related proteins (Figs. 1C and 1D).

Despite these similarities, the HRT proteins have several unique residues that distinguish them from Hairy-related bHLH factors. Unlike most bHLH proteins that interact with the canonical E box DNA binding site (CANNTG) (Murre *et al.,* 1989), Hairy proteins contain a conserved proline residue in the basic region and instead bind to an N box (CACNAG) (Ohsako *et al.,* 1994). The HRT proteins, however, share a common glycine residue instead of a proline at this critical site (Fig. 1B). In addition, Hairy family members are characterized by a highly unique and conserved 4-amino-acid region (WRPW) in the C-terminus that is involved in interaction with the Groucho-related family of corepressors (Fisher and Caudy, 1998b). Although HRT1 and HRT2 share similar motifs of YRPW and YQPW, respectively, they are distinct from that conserved in the Hairy family of proteins; moreover, HRT3 contains an even more distant YHSW sequence in this location. In contrast, the carboxy-terminal amino acids (TE(I/V)GAF) are highly conserved in the three HRT proteins (Fig. 1A), although this motif has not been characterized in any other bHLH transcription factors. Thus, while HRT1–3 share structural similarities to the Hairy family of proteins, they are clearly distinct and define a new subclass of Hairy-related bHLH transcription factors.

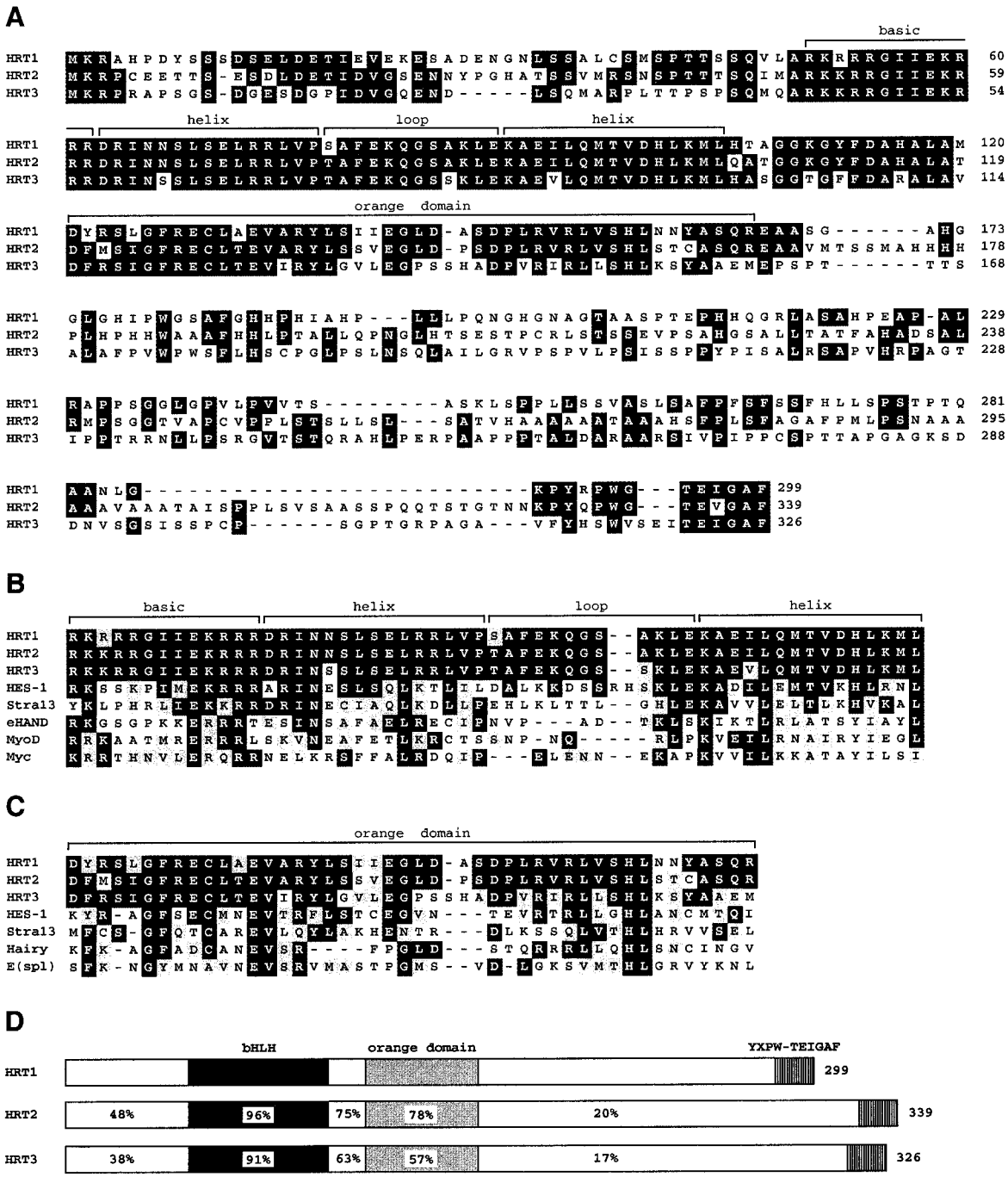


FIG. 1. Sequence alignment of HRT1, -2, and -3 and comparison with other bHLH transcription factors. (A) Deduced amino acid sequences of HRT1, -2, and -3. Identical amino acid sequences are shown in black boxes and functional domains are indicated in brackets. Amino acid numbers are shown on the right. cDNA sequences of *HRT1, -2,* and *-3* have been deposited with GenBank under Accession Nos. AF172286, AF172287, and AF172288, respectively. (B) Comparison of HRT bHLH domains with other bHLH transcription factors. Identical residues are shaded in black and similar residues shaded in gray. (C) Comparison of orange domains of HRT proteins with related transcription factors. Identical amino acids are shown in black, and similar residues are shaded in gray. (D) Model of protein domains with percentage identity of HRT2 and HRT3 with HRT1. Numbers of amino acids are shown on the right.

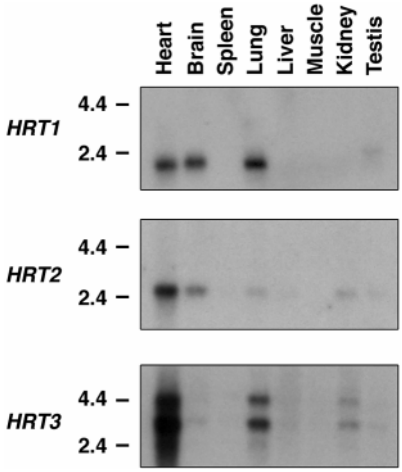


FIG. 2. mRNA expression of *HRT1, -2,* and *-3* in adult mouse tissues. Northern blot analysis of *HRT1, HRT2,* and *HRT3* on adult mouse poly(A)$^+$ RNA. Positions of RNA size markers are shown on the left in kb.

HRT1–3 Expression in Adult Mouse Tissues

In order to determine if *HRT1, HRT2,* and *HRT3* were expressed in a tissue-specific fashion, Northern blot analysis was performed on multiple mouse adult tissues. Each gene was distinct in size of transcript and pattern of expression (Fig. 2). *HRT1* mRNA (2.3 kb) was detected in the heart, brain, and lung. In contrast, 2.5-kb *HRT2* mRNA was most abundant in the heart but also detectable in the brain, kidney, and lung. Finally, *HRT3* was highly expressed in the heart with a lower level of expression in the lung and kidney. Although the predominant transcript detected was 3.0 kb, a band representing a 4.4-kb message also hybridized to the HRT3 probe, possibly representing an alternatively spliced form.

Embryonic Expression of HRT1–3

We also performed *in situ* hybridization analysis to define the expression patterns of *HRT1, -2,* and *-3* during mouse embryogenesis. Each gene was expressed in a very dynamic and often complementary fashion. The predominant sites of expression at E9.5 were in cardiac, somitic, and pharyngeal arch precursors: *HRT1* and *HRT2* in the atrial and ventricular precursors, respectively; *HRT1* and *HRT3* in the somitic precursors; *HRT1* and *HRT2* in the pharyngeal arches and clefts (Figs. 3A–3C). Because *HRT* gene expression was dependent upon the developmental stage, the evolving patterns of each in the heart, somites, and pharyngeal arches are compared in more detail below.

Cardiovascular Expression of HRT Genes

As early as the cardiac crescent stage (E7.75), precardiac mesodermal cells are fated to form either atrial or ventricular cells with the atrial precursors lying in the more posterior portion of the cardiac crescent. The early heart tube maintains this segmentation with the ventricular and atrial precursors segmented in an anterior–posterior fashion, respectively. *HRT1* and *HRT2* were expressed in the atrial and ventricular precursors, respectively, throughout cardiac development (Fig. 4). *HRT1* was expressed at low levels in the most posterior branches of the forming heart tube at E8.0–8.5 (Figs. 4A and 4B). These branches are known as the sinus venosa and later form the right and left atrial chambers of the heart (DeHaan, 1965). As heart development progressed, *HRT1* expression was restricted to the atrial segment of the looped heart posterior to the atrioventricular canal (Figs. 4C–4E). By radioactive *in situ* hybridization at E10.5, *HRT1* transcripts were abundant in the atrial chambers and epicardium, but were not detected in the right or left ventricle precursors (Fig. 3D).

In sharp contrast to *HRT1, HRT2* was expressed specifically in the anterior part of the primitive heart tube that is fated to form the ventricular segments (Figs. 4F and 4G). No *HRT2* transcripts were detected in the atrial precursors marked by *HRT1.* This pattern was maintained in the looped heart tube and a sharp border of expression was observed at the atrioventricular junction with *HRT2* mRNA detected only in the ventricles. Radioactive *in situ* hybridization revealed that *HRT2* transcripts were most abundant in the compact zone of the ventricles, although lower levels were detectable in the trabeculae (Fig. 3E). Unlike *HRT1* (Fig. 3D), *HRT2* was also expressed in the cushion mesenchyme of the outflow tract at low levels (Fig. 3E). Interestingly, ventricular expression of *HRT2* was maintained at E15.5 (Fig. 7B), while atrial expression of *HRT1* was downregulated at E15.5 (Fig. 7A). *HRT3* mRNA was not detected at comparable stages of heart development by whole-mount or section *in situ* hybridization (Figs. 3C, 3F, and 7C), even though *HRT3* is abundantly expressed in the adult heart (Fig. 2).

The *HRT* genes were also expressed in a distinct pattern in the embryonic vasculature. At E10.5, *HRT1* and *HRT2* were highly expressed in the dorsal aorta, aortic arch arteries, and aortic sac (Figs. 3D and 3E), but *HRT3* was weakly detected only in the dorsal aorta (Fig. 3E). In contrast, *HRT3* was most abundantly expressed in the aorta and pulmonary artery at E15.5 (Fig. 7C), while vascular expression of *HRT1* and *HRT2* was lower at E15.5 than E10.5 (Figs. 7A and 7B).

HRT Gene Expression in Developing Somites

Although the heart tube is segmented along the AP axis, the most obvious AP segmentation in vertebrates is at the trunk level where vertebrae, associated muscles, and the peripheral nervous system form in a metameric pattern (Tajbakhsh and Sporle, 1998). The embryonic origin of trunk segmentation lies in the formation of somites that bud off from the rostral presomitic mesoderm (PSM) and form in a rostrocaudal fashion (Gossler and Hrabe de

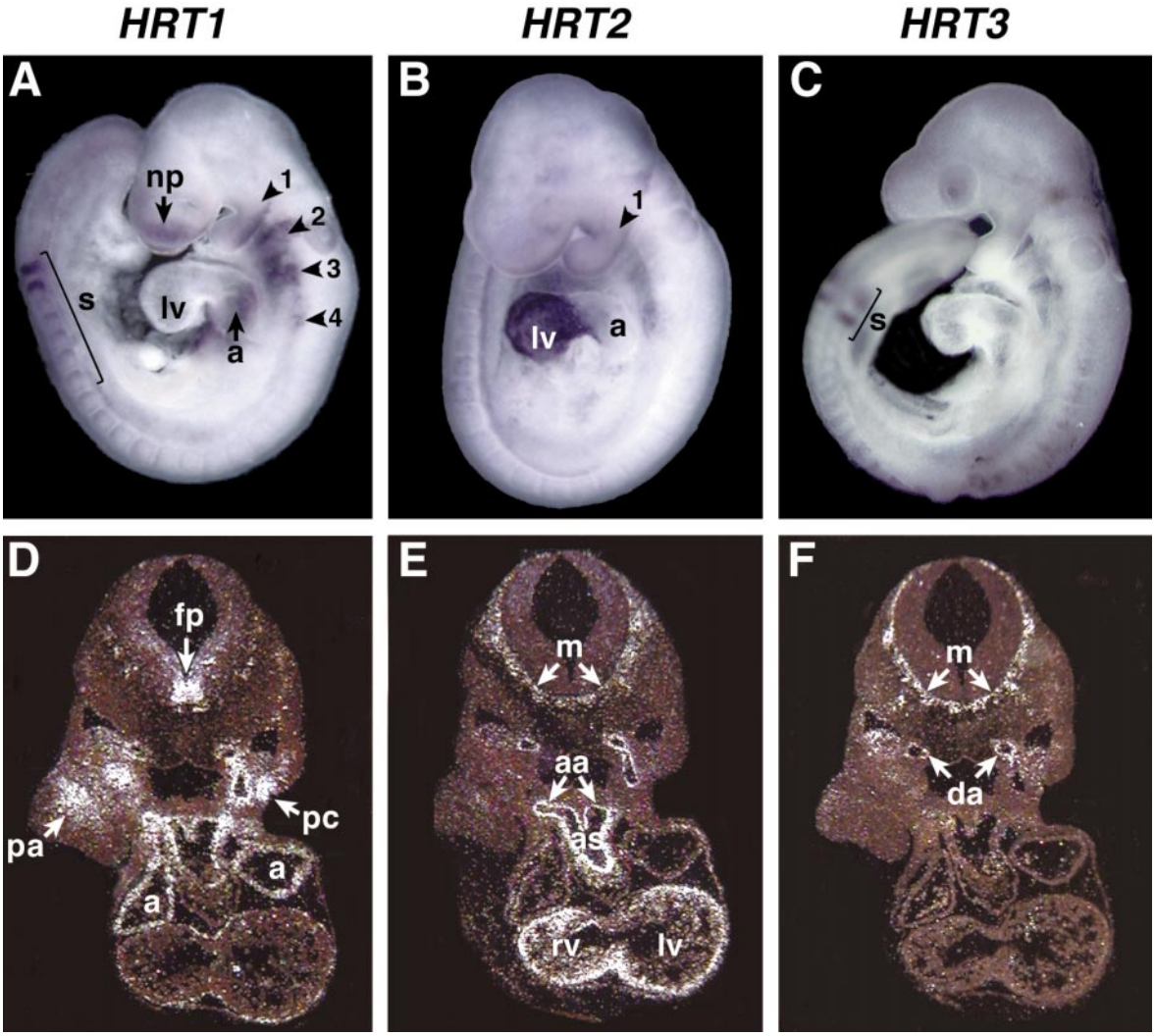
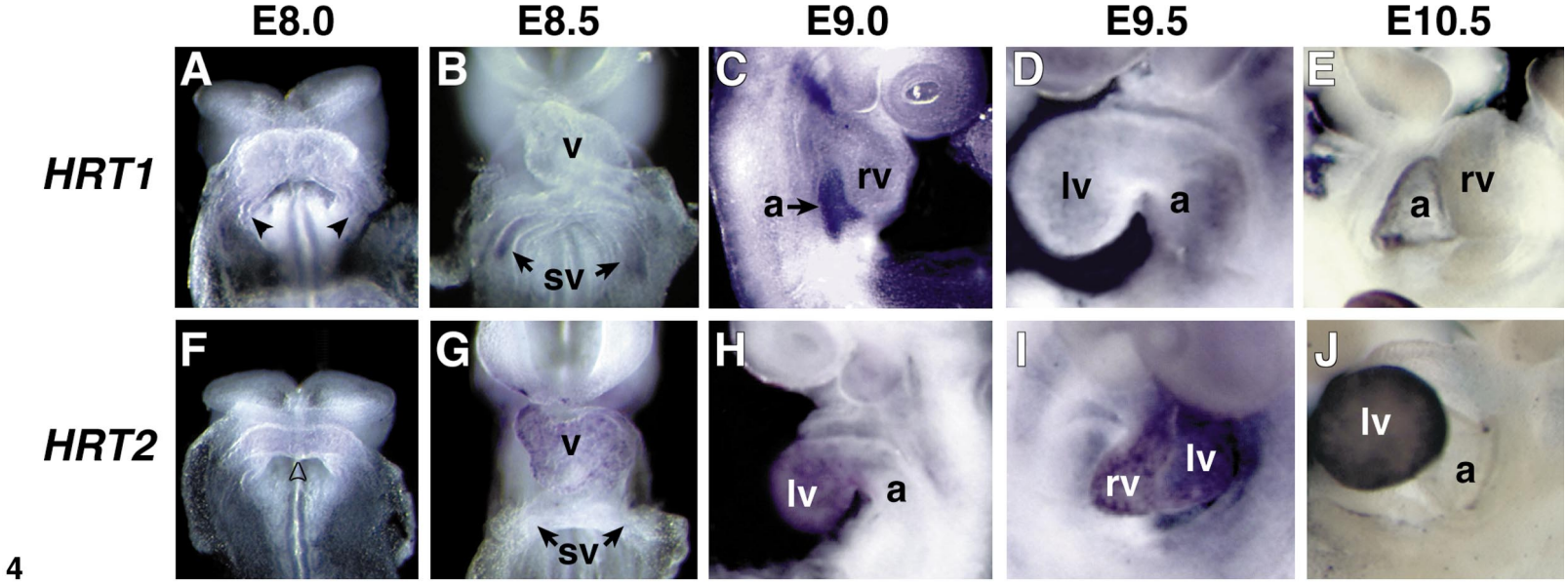


FIG. 3. Expression of *HRT1, -2,* and *-3* mRNA in mouse embryos. Whole-mount *in situ* hybridization of *HRT1* (A), *HRT2* (B), and *HRT3* (C) in E9.5 embryos, and radioactive section *in situ* hybridization of *HRT1* (D), *HRT2* (E), and *HRT3* (F) in E10.5 embryos. Left lateral views (A–C) or transverse sections (D–F) are shown. Arrowheads (numbered) indicate pharyngeal arches. a, atrium; aa, aortic arch arteries; as, aortic sac; da, dorsal aorta; fp, floor plate; lv, left ventricle; m, mesenchyme surrounding neural tube; np, nasal process; pa, pharyngeal arch; pc, pharyngeal cleft; rv, right ventricle; s, somites and presomitic mesoderm.

Angelis, 1998). *HRT1* was expressed in a strikingly dynamic pattern in the PSM and in somites. As early as E8.5, *HRT1* mRNA was detectable in two distinct bands, one in the PSM and the other in the caudal portion of somites that had already budded off the PSM (Fig. 5A). At E9.5, embryos at similar developmental stages displayed distinct expression patterns of *HRT1* (Figs. 5B–5D), suggesting that a broad domain of expression progressed rostrally in the PSM and resolved into the caudal portion of formed somites. This dynamic pattern of expression was reminiscent of chicken *hairy1* (c-hairy1), which shows a rhythmic periodicity of expression corresponding to each forming somite (Palmeirim *et al.,* 1997). *HRT3* was also expressed in a similar pattern (Figs. 5E and 5F), while *HRT2* expression was much weaker than that of *HRT1* or *HRT3.*

At E10.5, *HRT1* mRNA was detected in the rostral end of the PSM and recently formed somites, but *HRT2* and *HRT3* transcripts were not detectable by whole-mount *in situ* hybridization. Radioactive section *in situ* hybridization, however, revealed that the *HRT* genes were expressed in distinct domains within somites. *HRT1* was expressed predominantly in the dermomyotome. In contrast, *HRT2* transcripts were found in the paraxial mesoderm but not in the dermomyotome or sclerotome. Finally, *HRT3* was detected in the paraxial mesoderm and sclerotome, but not dermomyotome (data not shown).



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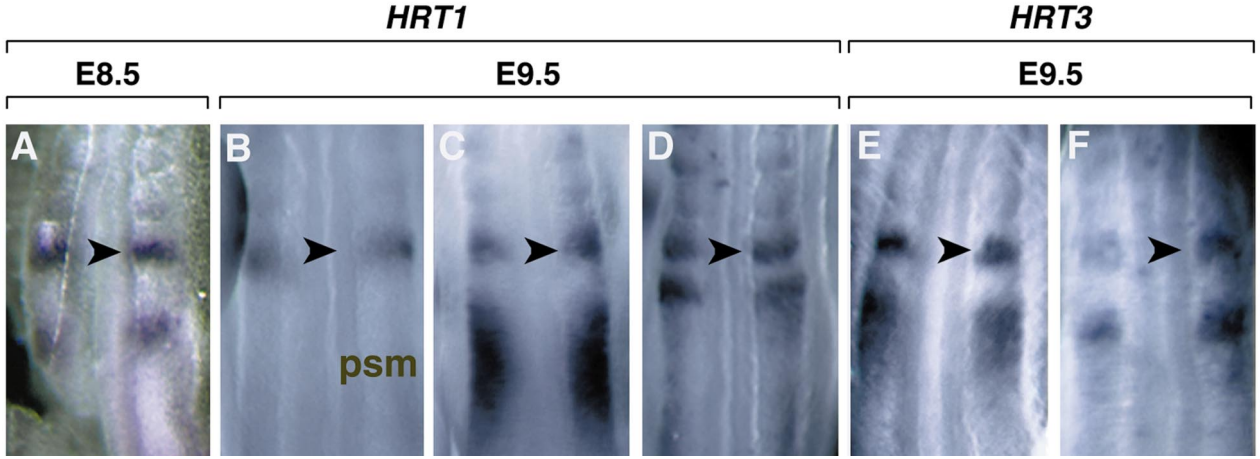

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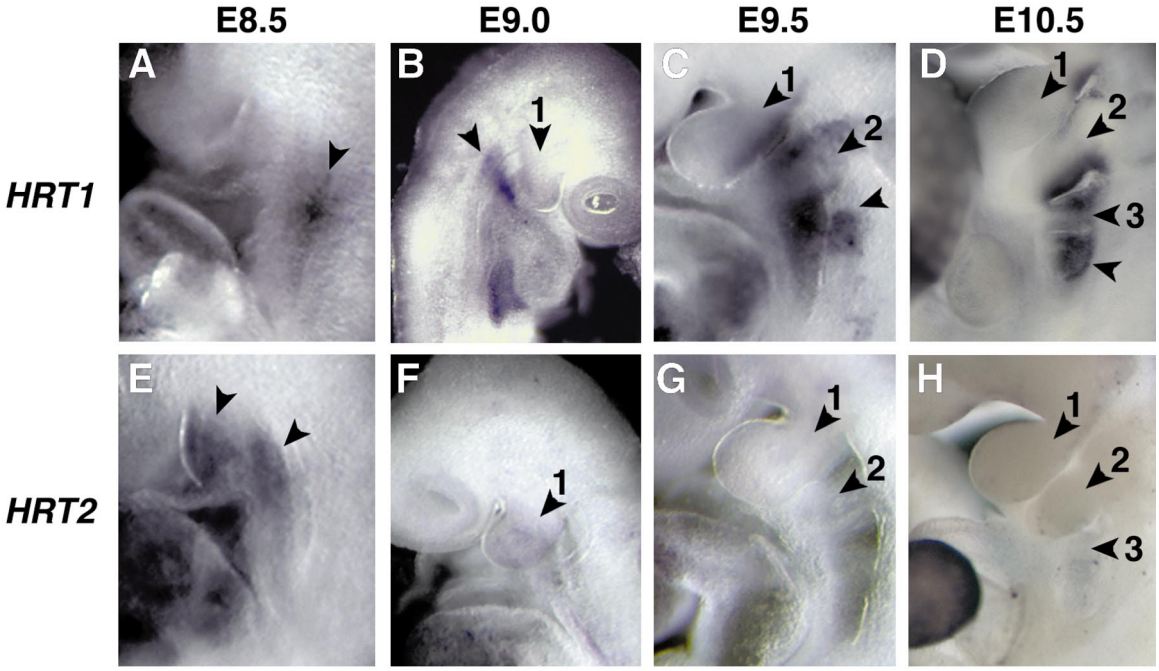
FIG. 4. Spatiotemporal expression pattern of *HRT1* and *HRT2* mRNA in the developing heart. Whole-mount *in situ* hybridization of *HRT1* (A–E) and *HRT2* (F–J). Frontal (A, B, F, and G), right lateral (C), and left lateral (D, E, H–J) views are shown at indicated embryonic days. Arrowheads indicate atrial precursors; open arrowhead indicates ventricular precursors; a, atrium; lv, left ventricle; rv, right ventricle; sv, sinus venosus; v, ventricle.

FIG. 5. Expression of *HRT1* and *HRT3* mRNA during somitogenesis. Whole-mount *in situ* hybridization of *HRT1* (A–D) and *HRT3* (E and F) at stages indicated. Arrowheads indicate newly formed somites. psm, presomitic mesoderm.



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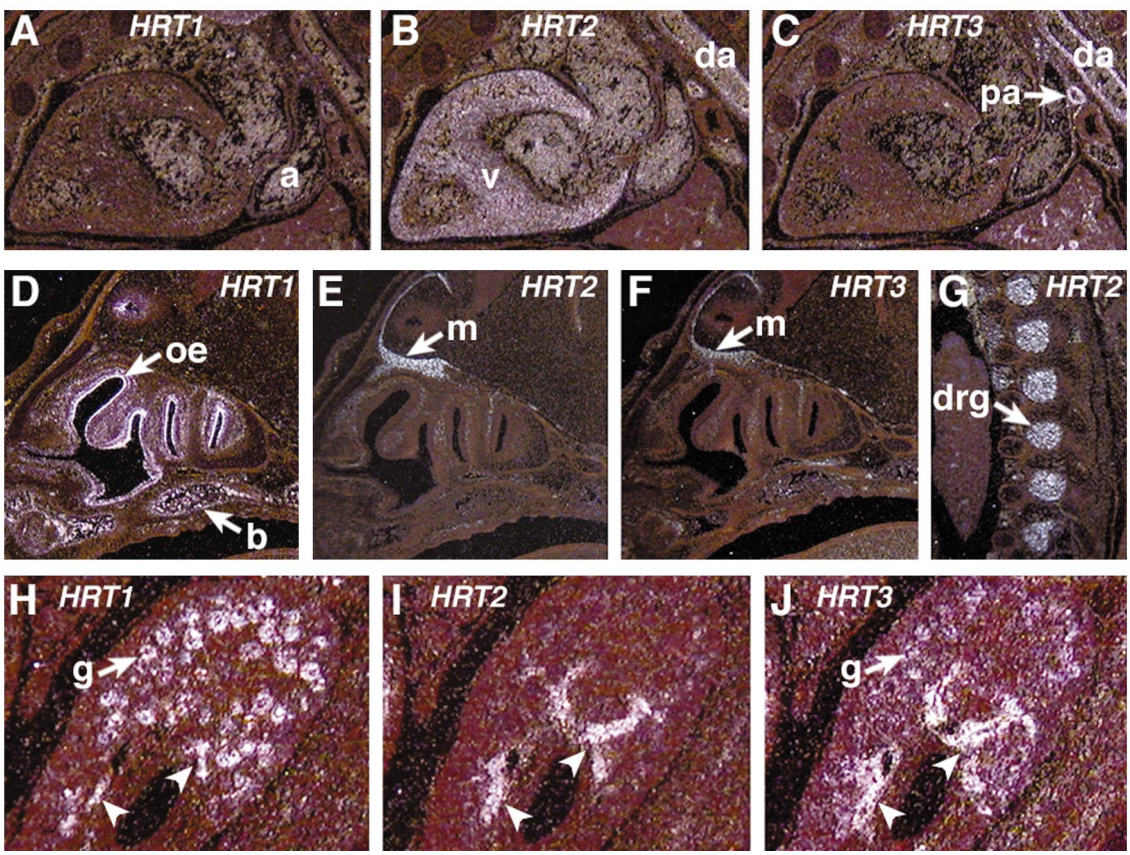

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FIG. 6. Expression of *HRT1* and *HRT2* in developing pharyngeal arches and pharyngeal clefts. Whole-mount *in situ* hybridization of *HRT1* (A–D) and *HRT2* (E–H). Lateral views of pharyngeal arch region of embryos are shown at indicated stages of development. Arrowheads indicate pharyngeal arches (numbered) or the regions expressing *HRT1* or *HRT2*.

FIG. 7. Expression of *HRT1*, *-2*, and *-3* in developing organs. Radioactive section *in situ* hybridization of *HRT1* (A, D, and H), *HRT2* (B, E, G, and I), and *HRT3* (C, F, and J) on sagittal sections of E15.5 embryos. Expression was observed in the heart, dorsal aorta (da), and pulmonary arteries (pa) (A–C); the olfactory epithelium (oe), mesenchyme surrounding the olfactory bulb (m), and dorsal root ganglia (drg) (D–G); the basisphenoid bone (b) (D); and the kidneys (H–J). Arrowheads indicate the mesenchyme surrounding the hylar vasculature in the kidney. a, atrium; g, glomerulus; v, ventricle.

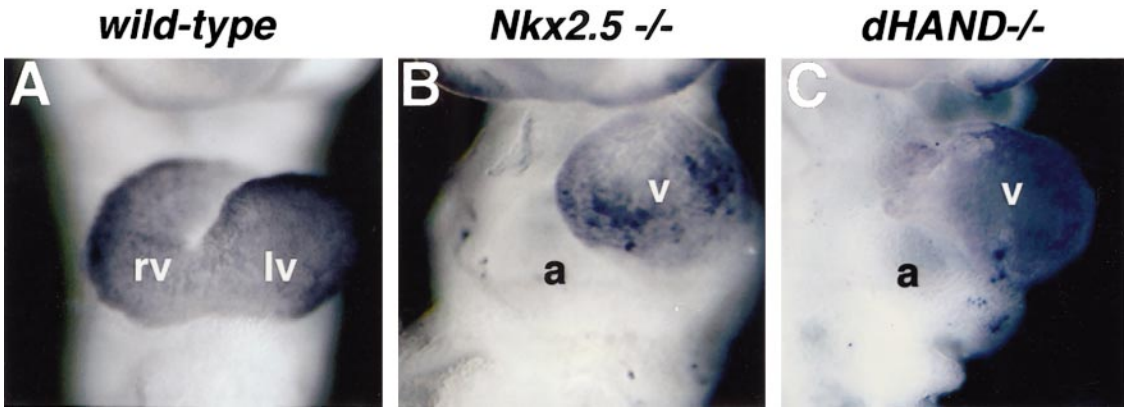

FIG. 8. Cardiac expression of *HRT2* mRNA in *Nkx2.5*-null or *dHAND*-null mutant. Frontal views of whole-mount *in situ* hybridization of *HRT2* on wild-type (A), *Nkx2.5*-null (B), and *dHAND*-null (C) embryos at E9.5. a, atrium; lv, left ventricle; rv, right ventricle; v, ventricle.

HRT Gene Expression in Pharyngeal Arch Precursors

Segmentation at the head level is most prominent in the rhombomeres and pharyngeal arches. Bilaterally symmetric pharyngeal arches form successively in a rostrocaudal fashion with the first pharyngeal arch becoming morphologically apparent at E8.5 (Kaufman, 1994). Neural crest cells migrate and populate the mesenchyme of each pharyngeal arch beginning at E9.0 and later differentiate into numerous craniofacial and neck structures (Serbedzija *et al.*, 1992). Once the first pharyngeal arch had formed, *HRT1* was not detectable there but was detected more caudally in the precursors of the second pharyngeal arch (Fig. 6A and 6B). Subsequently, its expression was observed in the third and fourth pharyngeal arch precursors as each arose (Fig. 6C and 6D). After each pharyngeal arch was morphologically apparent, *HRT1* expression was observed only in the intermediate region between the pharyngeal arches known as the pharyngeal clefts (Fig. 6C and 6D). Radioactive *in situ* hybridization on E10.5 embryos also revealed *HRT1* expression in the pharyngeal arches and clefts (Fig. 3D).

In contrast to *HRT1, HRT2* was expressed broadly around the first pharyngeal arch at E8.5 (Fig. 6E), and the expression became restricted to the first pharyngeal arch at E9.0 (Fig. 6F). The expression decreased rapidly and was not detectable in later embryos (Fig. 6G and 6H). *HRT3* expression was not detected in the pharyngeal arches at significant levels. Thus, *HRT1* and *HRT2* displayed distinct expression patterns in the pharyngeal arches, preceding pharyngeal arch formation sequentially along the AP axis.

Expression of HRT1–3 in Developing Organs

Radioactive *in situ* hybridization revealed distinct sites of *HRT* gene expression outside those described above. *HRT1* mRNA was expressed in the neural tube floor plate (Fig. 3D) and nasal process (not shown) at E10.5 and was abundant in the olfactory epithelium at E15.5 (Fig. 7D). In contrast, *HRT2* and *HRT3* transcripts were expressed in the mesenchyme surrounding the neural tube at E10.5 (Figs. 3E and 3F) and in the mesenchyme around the olfactory bulb at E15.5 (Figs. 7E and 7F). *HRT2* was also highly expressed in the dorsal root ganglion (Fig. 7G). Within the kidney, *HRT1* was highly expressed in the glomerular epithelium and in the mesenchyme surrounding the hylar vasculature (Fig. 7H). *HRT3* was also expressed in these two regions, but its glomerular expression was much weaker than that of *HRT1* (Fig. 7J). In contrast, *HRT2* expression was observed only around the vasculature (Fig. 7I). In addition, *HRT1* was expressed in the osteoblasts (Fig. 7D) and *HRT3* was detected in the paraspinous muscles (not shown). Thus, although the *HRT* genes shared expression in numerous organs, each was often expressed in distinct domains within individual tissues.

HRT Gene Expression in Cardiac Mutant Embryos

Targeted mutations of *Nkx2.5* and *dHAND* result in distinct cardiac defects (Lyons *et al.*, 1995; Srivastava *et al.*, 1997) that may represent abnormal development of ventricular segments of the heart. Thus, we examined expression of *HRT2,* a ventricle-specific marker, in *Nkx2.5* and *dHAND* mutant embryos to examine possible regulation of *HRT2* by these factors and the significance of Nkx2.5 and dHAND on chamber-specific development.

Embryos lacking Nkx2.5 fail to complete cardiac looping and exhibit downregulation of the ventricle-specific myosin light chain, MLC2V (Lyons *et al.*, 1995), and the left ventricle-specific marker, eHAND (Biben and Harvey, 1997). In contrast to these ventricular markers, *HRT2* transcripts were detected in the presumptive ventricular region of the *Nkx2.5* mutant heart tube, but not in the posterior atrial precursor region (Fig. 8B). *dHAND* mutants are thought to have a left ventricle and an atrial chamber (Srivastava *et al.*, 1997). Consistent with this, *HRT2* was

TABLE 1
Tissue-Specific Profiles of *HRT1, -2,* and *-3* Gene Expression

	HRT1	*HRT2*	*HRT3*
Heart			
Atrium/sinus venosus	+	−	−
Ventricle	−	++	−
Cardiac cushion	−	+	−
Adult heart	+	+	+
Vasculature			
Aortic sac	+	++	−
Aortic arch artery	++	+	−
Dorsal aorta	±	+	++
Pharyngeal arch			
Precursor	++	+	−
Cleft	++	−	−
Presomitic mesoderm	++	±	++

Note. −, ±, +, and ++ represent relative levels of expression of *HRT1, -2,* and *-3* in each organ. Levels of expression at E15.5 are shown for dorsal aorta.

expressed exclusively in the ventricular chamber and not in the more posterior chamber (Fig. 8C). These data indicate that the initial AP patterning of the heart primordia into ventricular and atrial precursors occurs normally in *Nkx2.5* and *dHAND* mutants. Furthermore, expression of *HRT2* in these mutants suggests that HRT2 does not function in regulatory pathways downstream of Nkx2.5 or dHAND.

DISCUSSION

Here we have described a new subfamily of bHLH proteins that possess structural features typical of Hairy family proteins but also share conserved amino acid residues not found in other Hairy family members. *HRT1, HRT2,* and *HRT3* exhibited distinct expression patterns with sharp boundaries in precursors of tissues patterned along the AP axis (summarized in Table 1).

Cardiac Chamber-Specific Expression of HRT Genes

Although the developing heart has unique segments that give rise to morphologically and functionally distinct chambers, few transcription factors are expressed in specific chambers. The homeodomain protein, Tbx5, is expressed in the left ventricle and atria (Bruneau *et al.,* 1999), while Irx4 is specific to the right and left ventricles (Bao *et al.,* 1999). The orphan nuclear receptor, COUP-TFII, was recently shown to be atrium-specific (Pereira *et al.,* 1999). In addition, dHAND and eHAND are expressed in the right and left ventricles, respectively, and in the outflow tract of the heart (Thomas *et al.,* 1998a). *HRT1* and *HRT2* are the only known regulatory genes to have complementary expression in the atrial and ventricular segments as early as the straight heart tube stage. Based on their expression in mutant mice, they do not appear to be downstream of the cardiac transcription factors, Nkx2.5, dHAND, or MEF2C (unpublished observations). It will be interesting to determine if HRT1 and HRT2 are involved in establishing regional gene expression, particularly in regulating *HAND* gene expression in specific segments of the heart.

Congenital heart defects (CHD) are the most common human birth defects. Most CHD affect specific regions or chambers of the heart, suggesting that disruption of segment-specific molecular pathways may underlie such CHD. The cloning and expression analysis of *HRT1* and *HRT2* in the atrial and ventricular chambers provide an entry to studying the role of Hairy-related signaling pathways, possibly involving Notch signaling, in cardiac development and CHD. It will be interesting to determine if HRT-related pathways are involved in conditions in which atrial-specific tissue extends into the region of the ventricle (Ebstein's anomaly), suggestive of a defect in establishing a clear boundary of atrial and ventricular-specific gene expression (Pavlova *et al.,* 1998). Finally, abnormalities in patterning of the neural crest-derived outflow tract and aortic arch arteries are common causes of CHD; dissection of cascades involving the HRT proteins may provide new insight into this dynamic process.

HRT Expression in Sites of Epithelial–Mesenchymal Interactions

Secreted signals, including fibroblast growth factors and bone morphogenetic proteins, from epithelial cells of the pharyngeal arches are necessary for proper development of underlying mesenchymal cells. Neural crest cells invade the pharyngeal arches and differentiate into mesenchymal cells that express numerous homeobox genes (Schilling, 1997; Kirby and Waldo, 1995) and the *HAND* genes (Thomas *et al.,* 1998b). *HRT1* and *HRT2* show their peak of expression in the pharyngeal arch precursors prior to neural crest invasion. For *HRT1,* this process is reiterated as each pharyngeal arch becomes sequentially specified and *HRT1* localizes in the pharyngeal clefts after neural crest cells populate the mesenchyme. In *dHAND* mutants, *HRT1* is expressed in pharyngeal arch precursors (unpublished observations) consistent with a later role for dHAND in the neural crest-derived component of the pharyngeal arch. It is notable that the *HRT* genes were expressed with intra-organ specificity in other common sites of epithelial–mesenchymal interactions, including the kidneys, limb buds, and vessels.

Expression of HRT Genes in Paraxial Mesoderm and Somites

Within the unsegmented paraxial mesoderm, there is a striking similarity between the expression patterns of *HRT1* and *HRT3* and components of the Notch–Delta

signaling system. Formation of somites, the basic unit of segmentation of the vertebrate body plan, occurs through progressive budding from the anterior end of the segmental plate mesoderm and requires three distinct events: prepatterning of the paraxial mesoderm, formation of boundaries between prospective somites, and somite differentiation (McGrew and Pourquie, 1998). Several lines of evidence suggest that somite formation is controlled by a regulatory system analogous to the Notch–Delta signaling system that controls cell fate decisions and boundary formation in the *Drosophila* embryo. *Delta* and *Notch* are expressed in complementary patterns in the presomitic and somitic mesoderm of vertebrate embryos (Conlon *et al.*, 1995). Similarly, bHLH transcription factors related to *Drosophila* Hairy and Enhancer of split (E(spl)) are expressed with a rhythmic periodicity in the unsegmented paraxial mesoderm, consistent with the notion that they establish a molecular "clock" that responds to Notch–Delta signaling to control vertebrate segmentation (Palmeirim *et al.*, 1997). Moreover, mice homozygous for null mutations in *Notch* (Swiatek *et al.*, 1994; Conlon *et al.*, 1995) or *Delta*-like (Kussumi *et al.*, 1998) genes show abnormalities in somite formation similar to those seen in mice lacking the Suppressor of hairless (Su(H)) homolog RBP-JK (Oka *et al.*, 1995). Ectopic expression of antimorphic forms of X-Delta-2 and Su(H) in *Xenopus* embryos also perturbs segmentation (Jen *et al.*, 1997). Based on the similarity in expression patterns of *HRT* and *HES* genes in the paraxial mesoderm and the amino acid conservation within the bHLH regions of these factors, it is tempting to speculate that *HRT* genes will participate in somite segmentation. The bHLH transcription factor Paraxis is also expressed in the unsegmented paraxial mesoderm and in somites (Burgess *et al.*, 1995), but it does not show the striped pattern characteristic of *HRT* gene expression. In *paraxis*-null mice, the paraxial mesoderm becomes segmented, but epithelial somites fail to form. *HRT1* was expressed in a normal pattern in the presomitic region of *paraxis* mutant embryos (unpublished observations), indicating that it is independent of Paraxis.

bHLH Transcription Factors as Targets for Notch–Delta Signaling

In *Drosophila*, interaction of Delta with its receptor, Notch, on adjacent cells results in selection of specific cells from a precursor population and establishment of cell boundaries through a process known as lateral inhibition (McGrew and Pourquie, 1998). Activation of the Notch receptor results in proteolytic cleavage of its carboxyl-terminus, which translocates to the nucleus where it interacts with the DNA binding protein, Su(H). The Notch–Su(H) complex activates transcription of the *Hairy/E*(*spl*) genes, which encode inhibitory bHLH proteins. All components of this signaling system have been identified in vertebrates, and members of the Hairy/HES family of bHLH proteins have been shown to be upregulated in vertebrate cells by Notch signaling. However, Hairy-related pathways have not been shown to play a role in cardiac or pharyngeal arch development. Whether the HRT proteins are regulated by Notch signaling and function in a similar manner remains to be determined. However, it is interesting that human mutations in the Notch ligand, Jagged1, are responsible for Alagille syndrome and associated cardiac outflow tract defects (Oda *et al.*, 1997). Elucidation of the pathways regulated by the HRT proteins may provide insight into many features of normal and abnormal embryonic development that underlie many congenital birth defects.

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