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The bHLH Factors, dHAND and eHAND, Specify Pulmonary and Systemic Cardiac Ventricles Independent of Left–Right Sidedness

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dHAND and eHAND are basic helix-loop-helix transcription factors that play critical roles in cardiac development. The *HAND* genes have a complementary left–right cardiac asymmetry of expression with *dHAND* predominantly on the right side and *eHAND* on the left side of the looped heart tube. Here we show that although *eHAND* is asymmetrically expressed along the anterior–posterior and dorsal–ventral embryonic axes, it is symmetrically expressed along the left–right axis at early stages of embryonic and cardiac development. After cardiac looping, *dHAND* and *eHAND* are expressed in the right (pulmonary) and left (systemic) ventricles, respectively. The left–right (LR) sidedness of *dHAND* and *eHAND* expression is demonstrated to be anatomically reversed in situs inversus (*inv*/*inv*) mouse embryos; however, *dHAND* expression persists in the pulmonary ventricle and *eHAND* in the systemic ventricle regardless of anatomic position, indicating chamber specificity of expression. Previously we showed that *dHAND*-null mice fail to form a right-sided pulmonary ventricle. Here mice homozygous for the *dHAND* and *inv* mutations are demonstrated to have only a right-sided ventricle which is morphologically a left (systemic) ventricle. These data suggest that the *HAND* genes are involved in development of segments of the heart tube which give rise to specific chambers of the heart during cardiogenesis, rather than controlling the direction of cardiac looping by interpreting the cascade of LR embryonic signals.

INTRODUCTION

Pattern formation during embryogenesis is established along the anterior–posterior (AP), dorsal–ventral (DV), and left–right (LR) axes. The morphogenetic movements of the developing heart tube provide a unique model in which to study differential gene expression along all three embryonic axes. Classic embryologic studies indicate that bilaterally symmetric cardiac progenitor pools derived from the lateral mesoderm fuse at the ventral midline to form a linear heart tube (Yutzey and Bader, 1995). Cell fate analyses demonstrate that the straight heart tube is patterned in an AP fashion to form the cardiac outflow tract (conotruncus), right ventricle, left ventricle, and atria, respectively (reviewed in Olson and Srivastava, 1996). A cascade of asymmetric LR signals culminates in a highly conserved rightward looping of the heart tube which is the first morphologic evidence of LR embryonic asymmetry (Levin, 1997). Rightward looping begins to establish the spatial orientation of the four-chambered heart such that the pulmonary ventricle, which pumps blood to the lungs, lies on the right and the systemic ventricle, which pumps to the body, lies on the left. Disturbances of LR patterning in humans can result in visceroatrial heterotaxy syndrome and are often accompanied by a myriad of congenital cardiac defects related to abnormal cardiac looping (Gebbia *et al.*, 1997; Srivastava, 1997b).

Insights into the signals controlling LR asymmetry have recently come from chick and mouse studies. In the chick, asymmetric left-sided expression of nodal, a transforming growth factor β (TGFβ) family member, along the lateral mesoderm is activated by left-sided expression of sonic hedgehog (shh) (Levin *et al.*, 1995) and is associated with rightward looping of the heart tube. Similar left-sided nodal expression has been demonstrated in Xenopus (Lohr *et al.*, 1997). Stimulation of activin receptor IIA, which is asym-

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metrically expressed along the right side of the chick embryo, inhibits shh expression on the right (Levin *et al.*, 1995). A snail-related zinc finger transcription factor (cSnR-1) is subsequently expressed asymmetrically in the right lateral mesoderm and plays a role in LR asymmetry as well (Isaac *et al.*, 1997).

In mice, a model of situs inversus (*inv*/*inv*), generated by random insertion of a tyrosinase gene into a locus on chromosome 4 (Yokoyama, *et al.*, 1993), is characterized by consistent reversal of visceral left–right asymmetry (cardiac, gastrointestinal, and pulmonary) during embryogenesis. The *inv* gene has yet to be identified but reversal of the asymmetric expression of nodal and lefty, another member of the TGFβ family, is associated with leftward looping hearts (Lowe *et al.*, 1996; Collignon *et al.*, 1996; Meno *et al.*, 1996). Thus far, the genes which show LR asymmetry of expression prior to heart looping are not expressed in the cardiac ventricular mesoderm and presumably specify the direction of looping through extracellular signals.

Although most genes are expressed homogeneously throughout the straight heart tube, AP patterning of the developing heart tube precedes cardiac looping. After cardiac looping, many cardiac-specific genes show regionalized expression (Lyons, 1994), while others are regulated by *cis* elements in a chamber-specific fashion (Ross *et al.*, 1996; Kuisk *et al.*, 1996; Kelly *et al.*, 1995; reviewed in Firulli and Olson, 1997). The regulatory factors which control the AP patterning and subsequent chamber specification of the developing heart remain unknown.

Transcription factors sharing a basic helix-loop-helix (bHLH) motif serve as critical regulatory proteins for diverse cell types including neuronal (Jan and Jan, 1993; Lee *et al.*, 1996), hematopoietic (Shivdasani *et al.*, 1995), and skeletal muscle (Olson and Klein, 1994) cells. We have described the expression of two closely related bHLH genes, *dHAND* and *eHAND*, also known as *Hed*/*Thing2* and *Hxt*/*Thing1* (Cserjesi *et al.*, 1995; Cross *et al.*, 1995; Hollenberg *et al.*, 1995; Srivastava *et al.*, 1995), respectively, during embryogenesis and demonstrated a critical role for the *HAND* genes during cardiac looping in the chick (Srivastava *et al.*, 1995). During mouse cardiogenesis, *dHAND* and *eHAND* are expressed in a complementary fashion in the future right and left ventricles, respectively (Srivastava *et al.*, 1997). Targeted deletion of the *dHAND* gene in mice demonstrated a requirement for dHAND in the development of cells fated to form the future right ventricle during the period of cardiac looping (Srivastava *et al.*, 1997). Although eHAND's role during cardiogenesis remains unclear, it has been implicated as a mediator of the LR signaling pathway resulting in rightward looping of the heart tube. This inference is based upon demonstration of a LR asymmetry in eHAND expression at the caudal end of the straight heart tube prior to cardiac looping (Biben and Harvey, 1997).

Here we examine the asymmetry of *dHAND* and *eHAND* gene expression in more detail along all three embryonic axes and utilize the *dHAND*-null and situs inversus mouse models to determine the relationship of the *HAND* genes to development of embryonic asymmetry. In contrast to the LR asymmetry reported by Biben and Harvey (1997), we demonstrate symmetric LR expression of eHAND in the caudal region of the straight heart tube. Maintenance of the unique AP and DV pattern in the situs inversus (*inv*/*inv*) mouse model is shown with subsequent reversal along the LR axis, suggesting that the *HAND* genes respond to signals along the AP and DV axes which are translated later to asymmetries along the LR axis. Finally, the notion of dHAND and eHAND as chamber-specific, rather than side-specific factors is further supported by the finding of a hypoplastic pulmonary ventricle in mice harboring homozygous mutations in both *dHAND* and *inv* genes, just as was seen in *dHAND*-null mice, although the hypoplastic ventricle is left-sided in the double mutant.

MATERIALS AND METHODS

Embryo Collection and Genotyping

C57BL6 inbred mice were used for wild-type embryo collection. Heterozygous *inv* mice were intercrossed to obtain embryos homozygous for the *inv* gene (*inv*/*inv*). Embryos were harvested from wild-type and *inv*/*inv* pregnant females at time points ranging from E8.0 to E11.5 and were fixed overnight in 4% paraformaldehyde/phosphate-buffered saline solution. For embryos examined prior to cardiac looping, yolk sac DNA was obtained for genotyping by polymerase chain reaction (PCR) to identify *inv*/*inv* embryos. Primers used for PCR correspond to a region of the genome that is deleted at the *inv* locus and are as follows: CTGTCCAGTGCACCATCTGGACCTC and GATTACGTAATAGTGGTCCCTCAGG. Homozygous *inv*/*inv* embryos were identified based on the absence of a PCR amplification band. Embryos were stored in 70% ethanol at −20°C before being used for *in situ* hybridization.

In Situ Hybridization

Whole-mount *in situ* hybridizations were performed using digoxigenin-labeled antisense riboprobes synthesized from full-length dHAND and eHAND cDNAs. The dHAND cDNA was linearized with *Eco*RI and SP6 RNA polymerase used to synthesize the riboprobe. The eHAND cDNA was linearized with *Not*I and T7 RNA polymerase and used for riboprobe synthesis. *In situ* hybridizations were performed as previously described (Srivastava *et al.*, 1995). Briefly, embryos were prehybridized in hybridization buffer without probe at 60°C for 3 h; digoxygenin-labeled riboprobes were added and incubated at 60°C for 18 h. After a series of washes, embryos were incubated with alkaline phosphatase-conjugated anti-digoxygenin antibodies at room temperature for 1 h. Following another series of washes, embryos were incubated in a substrate color reaction mixture (Boehringer #1442074) for 12 h in darkness. Color reaction was terminated by fixing embryos in 4% paraformaldehyde, 0.1% glutaraldehyde.

Generation of dHAND and *inv* Double Mutants

Mice heterozygous null for the *dHAND* gene in the C57BL6/SV129 background were intercrossed with *inv* heterozygous mice

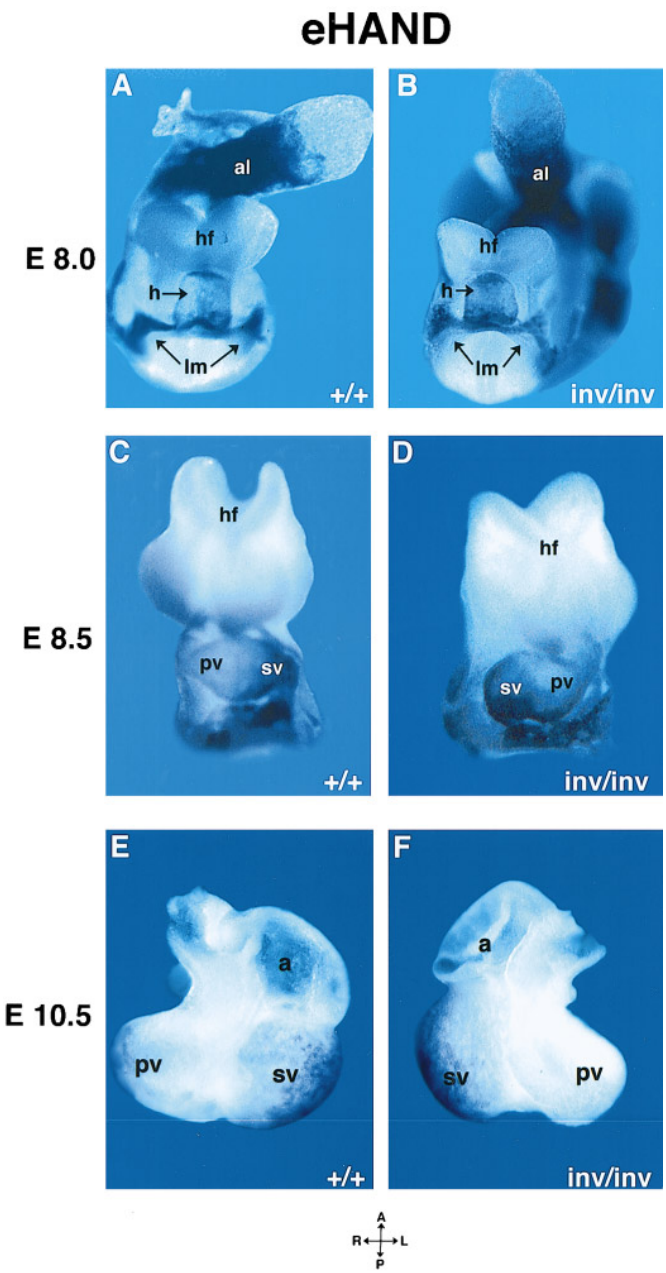

FIG. 1. *eHAND* expression in wild-type and *inv*/*inv* embryos. In a frontal–ventral view of mouse embryos at E8.0, *eHAND* expression is restricted to the anterior and posterior segments of the straight heart tube (A). No left–right asymmetry is seen in wild-type or *inv*/*inv* embryos (A, B) at the level of the heart (h) or lateral mesoderm (lm). Expression is seen in the allantois (al). As the heart tube begins to loop (E8.5), *eHAND* expression becomes left-sided in wild-type and right-sided in *inv*/*inv* embryos (C, D). The posterior regions of embryos in C and D were removed for a clearer ventral view. Later (E10.5), *eHAND* expression is restricted to the systemic ventricle (sv) in wild-type and *inv*/*inv* hearts, although the left–right orientation is reversed (E, F). Some *eHAND* expression is seen on the outside edge of the pv of wild-type and *inv* hearts, although this is not visible in the photographic plane of the *inv* heart (F). Hearts were dissected free from embryos to show the rightward loop (wild-type, E) and leftward loop (*inv*/*inv*, F) in frontal views. hf, head fold; a, atrium; pv, pulmonary ventricle.

in the FVB background. From their offspring, genotyping identified males and females heterozygous for both the *dHAND* and *inv* mutations. Intercrossing of such double heterozygotes resulted in pregnancies which were terminated by cesarean section at E9.5. Yolk sacs of embryos were genotyped for *dHAND* as previously described (Srivastara *et al.*, 1997) and *inv* loci as described above to identify embryos homozygous null for the *dHAND* and *inv* loci.

Histology

Wild-type and *inv*/*inv* embryos hybridized to the eHAND riboprobe were embedded in paraffin after fixation. Transverse sections were made at 5-μm intervals throughout the embryo. Paraffin was cleared in xylene and photographs of sections were taken without counterstaining. Wild-type, *dHAND*-null, and *dHAND*/*inv* double mutant embryos were fixed and embedded in paraffin. Transverse sections were made at 5-μm intervals and counterstained with hematoxylin and eosin.

RESULTS

In light of the critical role of the *HAND* genes during cardiogenesis and the asymmetric requirement of dHAND in the right versus left ventricle (Srivastava *et al.*, 1997), a detailed analysis of *dHAND* and *eHAND* expression was done. Mouse embryos with situs solitus (rightward looping hearts; wild-type) and situs inversus (leftward looping hearts; *inv*/*inv*) were compared and contrasted in order to determine the relationship of AP and LR cardiac asymmetry with respect to dHAND and eHAND expression and chamber specification.

Early LR Symmetry of eHAND Expression

Both *dHAND* and *eHAND* were uniformly expressed in the lateral mesoderm where cells fated to form the heart are arranged in a crescent shape at embryonic day 7.5 (E7.5) (Srivastava *et al.*, 1997; Biben and Harvey, 1997). Unlike *nodal*, which is expressed asymmetrically along the left lateral mesoderm and is implicated in controlling the direction of cardiac looping, mRNAs of both *HAND* genes were detected symmetrically along the left and right lateral mesoderm at E7.5 and E8.0 (Fig. 1A; Srivastava *et al.*, 1997). The expression of *eHAND* became restricted along the heart tube with interrupted expression seen in the anterior and posterior segments of the straight heart tube. The anterior expression marks the region fated to form the conotruncus, while the posterior segment marks the future left ventricle; the intervening right ventricle-forming region does not express *eHAND*. The expression of *eHAND* appears to be symmetric along the LR axis, but asymmetric along the AP axis in the straight heart tube.

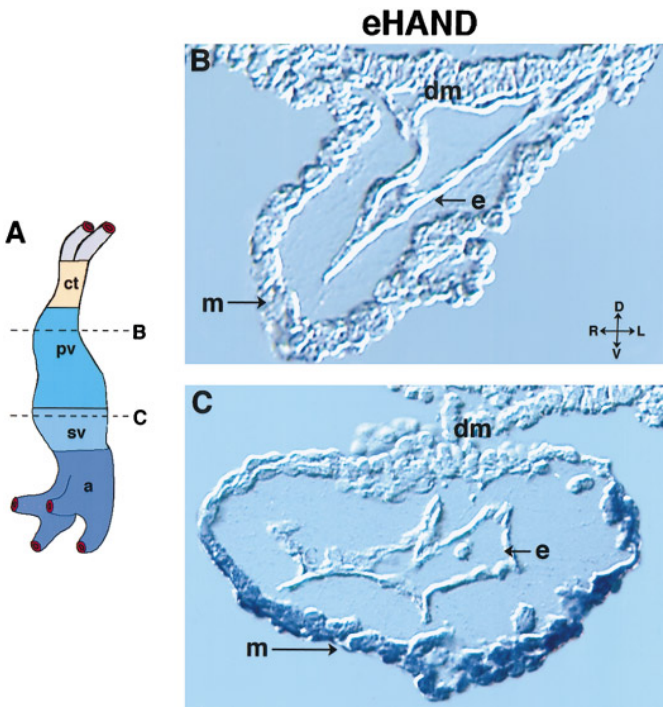

FIG. 2. Left–right (LR) symmetry of *eHAND* expression in the straight heart tube. Levels of sections in the straight heart tube are shown schematically (A). In transverse sections of an E8.0 heart, absence of *eHAND* expression is seen at the level of the future right (pulmonary) ventricle (pv) (B). More posteriorly, at the level of the future left (systemic) ventricle (sv), *eHAND* expression is symmetric on the left and right sides of the straight heart tube. Expression is apparent on the ventral (v) myocardium, but not the dorsal (d) surface of the heart tube (C). The endocardium (e) does not express *eHAND* and expression is restricted to the ventral myocardial (m) surface, with no expression seen on the dorsal surface. dm, dorsal mesocardium; ct, conotruncus; a, atria.

To confirm this and to determine if there were asymmetries of *eHAND* expression along other axes, histologic sections of 5 E8.0 embryos, which have a straight heart tube, were analyzed. In the region of the future right (pulmonary) ventricle, no expression of *eHAND* was evident (Fig. 2B). More posteriorly, in the region fated to form the left (systemic) ventricle, *eHAND* was expressed in a symmetric fashion along the LR axis (Fig. 2C) of each of the embryos sectioned. This was in contrast to the left-sided expression of *eHAND* suggested previously by Biben and Harvey (1997) at a similar level of section in the straight heart tube. At E8.0, *eHAND* was expressed on the ventral but not dorsal surface of the heart tube (Fig. 2C). This finding provides early molecular evidence of DV asymmetry in the developing heart. The ventral surface is directly apposed to the pericardium, which also expresses both *dHAND* and *eHAND*, raising the possibility of interaction between the two cell types.

eHAND expression in the ventricle became left-sided after the heart tube initiated a rightward loop at E8.5 (Figs. 1C and 1E). This sidedness appears to be a consequence of cardiac looping as the interrupted pattern established in the straight heart tube is maintained and converted to a LR asymmetry. Within the atria, expression diminishes after looping and is only seen in symmetrical streaks along the atrial wall (Fig. 3A). The atrial symmetry is in contrast to the asymmetrical atrial expression suggested by Biben and Harvey (1997).

eHAND Marks the Systemic Ventricle in Wild-Type and inv/inv Embryos

To determine if *eHAND* would be expressed in a cardiac chamber-specific fashion rather than a LR-specific fashion, *eHAND* expression in *inv/inv* mice was examined. When the straight heart tube forms a leftward, instead of rightward, loop in *inv/inv* embryos, the morphologic right (pulmonary) ventricle assumes a left-sided anatomic position. Similarly, the morphologic left (systemic) ventricle develops on the anatomic right side of the embryo. Similar to that seen in the wild-type, *eHAND* was expressed in an interrupted fashion along the straight heart tube of *inv/inv* embryos (Fig. 1B). However, as the heart tube formed a leftward loop in *inv/inv* embryos, *eHAND* expression assumed a right-sided location, in contrast to the normally left-sided expression (Figs. 1C and 1D; Figs. 3A and 3B). eHAND mRNA continued to be detected in the region fated to form the systemic ventricle, which is connected to the venous inflow (atria) in the wild-type and *inv/inv* embryos (Figs. 1E and 1F; Figs. 3A and 3B), although the anatomic location of this ventricle was reversed in the two. These findings are consistent with the LR symmetry and AP asymmetry of *eHAND* expression at the straight heart tube stage and suggest that *eHAND* expression is chamber-specific rather than side-specific.

Histological analysis of *eHAND* expression in wild-type and *inv/inv* embryos confirmed the expression of *eHAND* in the systemic ventricle of both types of embryos, although in reversed anatomical locations (Figs. 4B and 4D). *eHAND* expression was limited to the myocardial layer of the heart tube with no expression in the endocardial layer. Furthermore, expression of *eHAND* was seen on the greater (outer) curvature of the looped heart tube but not the lesser (inner) curvature, regardless of the direction of looping. This difference is likely related to the initial dorsal–ventral asymmetry of *eHAND* expression where *eHAND* is expressed only on the ventral aspect of the straight heart tube. Torsional effects of cardiac looping likely result in the dorsal part of the heart tube assuming the inner curvature of the looped heart while the ventral aspect forms the outer curvature. The dynamic alterations in *eHAND* expression pattern suggest this type of morphologic movement.

dHAND Marks the Pulmonary Ventricle in Wild-Type and inv/inv Embryos

dHAND expression was, in contrast to *eHAND* expression, seen uniformly throughout the straight heart tube and

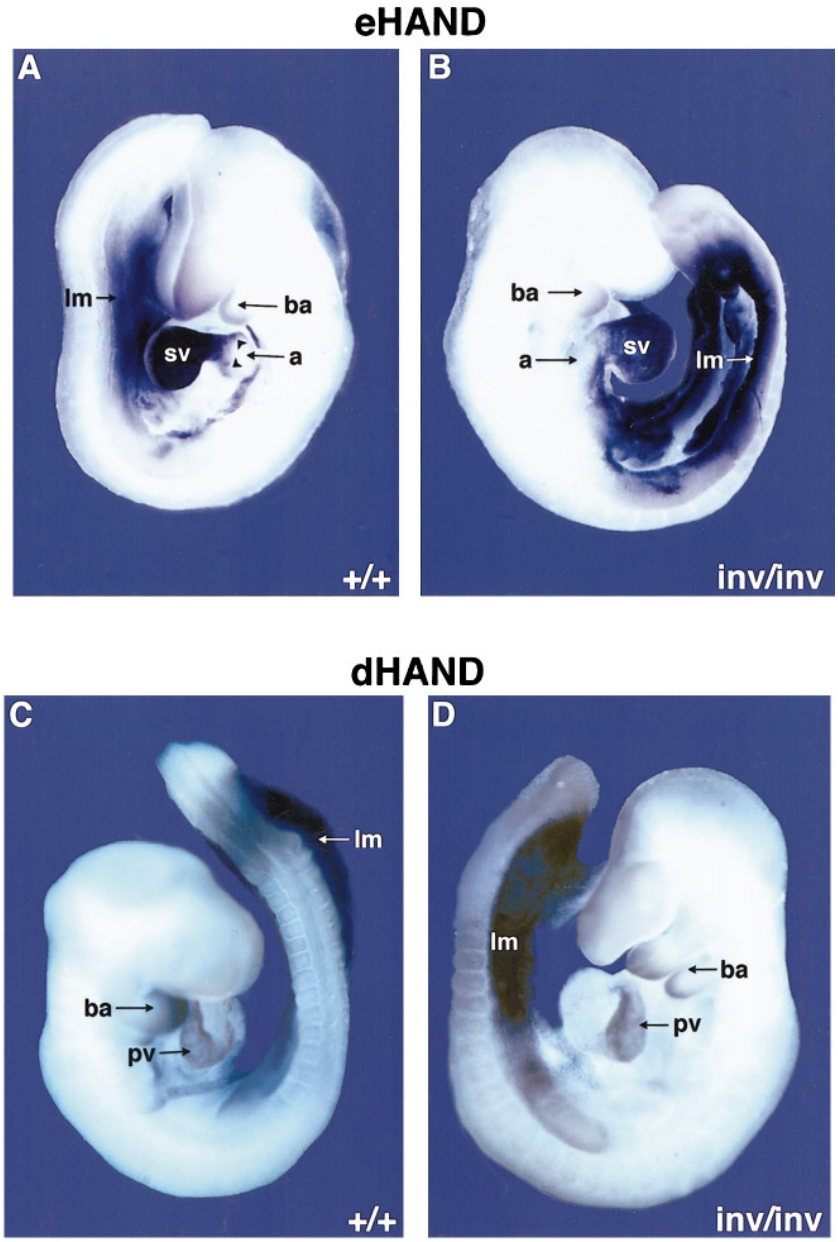

FIG. 3. eHAND and dHAND mark the systemic and pulmonary ventricles. In lateral views of E9.5 embryos, left–right reversal of *eHAND* (A, B) and *dHAND* (C, D) expression in wild-type (+/+) and *inv*/*inv* embryos is seen. *dHAND* expression is maintained in the pulmonary ventricle (pv) in *inv*/*inv* embryos, although this ventricle is now on the left side of the embryo (C, D). *eHAND* expression is seen on the right side of *inv*/*inv* hearts, but is still in the systemic ventricle (sv) as seen in wild-type. Streaks of *eHAND* expression (arrowheads) are seen in the upper and lower edges of the atria (a) near the atrioventricular junction but not in the body of the atria (A, B). Branchial arch (ba) and lateral mesoderm (lm) expression of eHAND and dHAND is seen (A–D).

became restricted to the right side of the rightward looped heart as previously described (Srivastava *et al.*, 1997). To determine if the cardiac asymmetry of *dHAND* expression correlated with the LR axis of the embryo or if it was chamber-specific, the expression of *dHAND* in situs inversus (*inv*/*inv*) mice was examined. *dHAND* expression was anatomically reversed in *inv*/*inv* embryos and was seen on the left side of the looped heart tube (Figs. 3C and 3D). Morphologically, however, *dHAND* expression persisted in the region fated to form the future pulmonary ventricle, which is connected to the outflow vessel of the heart (truncus arteriosus) and has a characteristic morphology. This find-

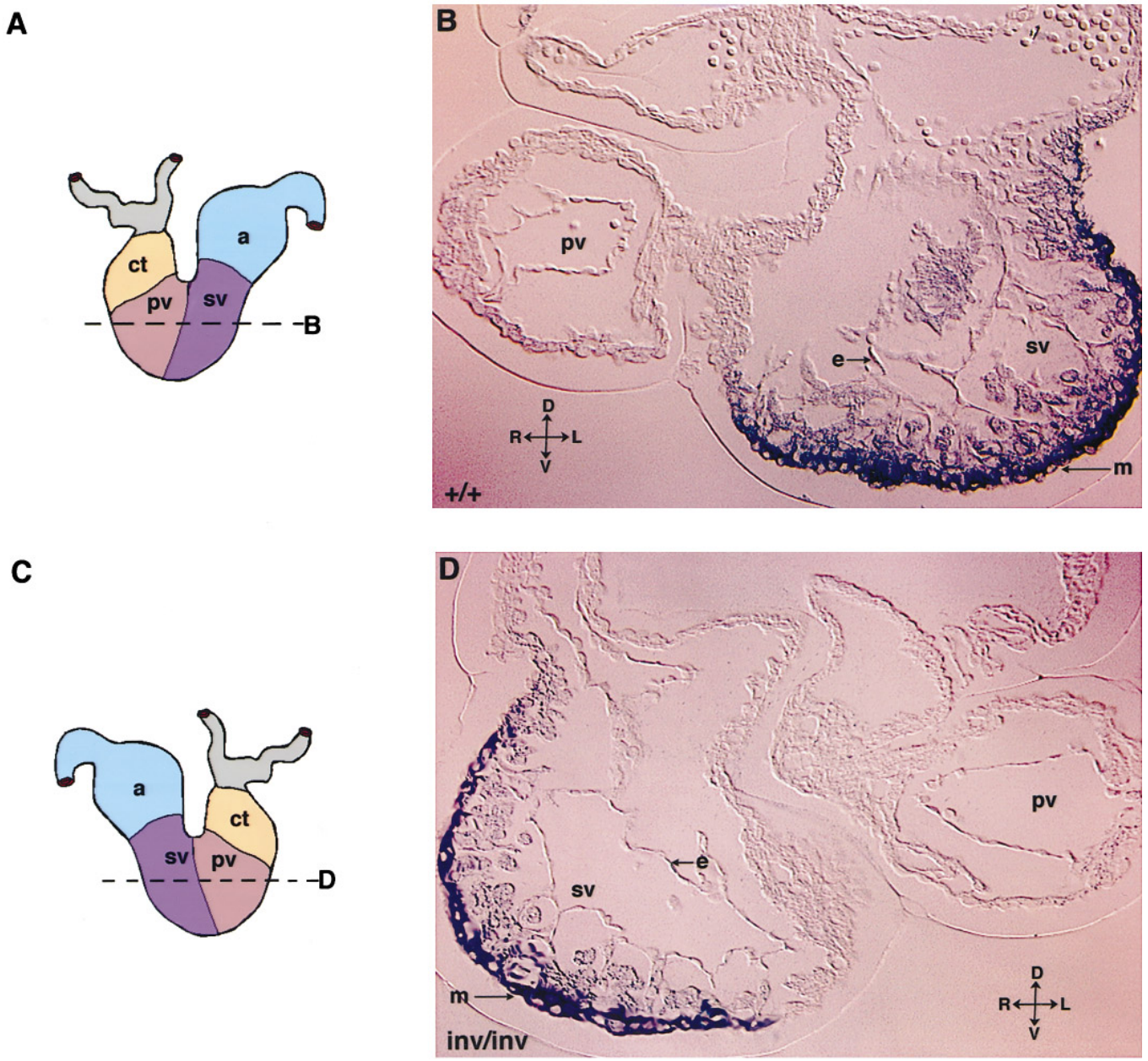

FIG. 4. Histologic analysis of *eHAND* expression in wild-type and *inv*/*inv* hearts. Planes of section through the looped heart tube are shown schematically (A, C). *eHAND* expression in wild-type (+/+) and *inv*/*inv* E9.5 embryos was analyzed by transverse thin sections. In wild-type embryos, *eHAND* expression was apparent in the myocardium of the left-sided systemic ventricle (sv), but not the right-sided pulmonary ventricle (pv) (B). In *inv*/*inv* embryos, the myocardium (m) of the systemic ventricle continued to express *eHAND*, although now located on the right side of the heart (D). Note restriction of expression to the outer curvature of the ventricle compared to the inner curvature. ct, conotruncus; a, atria; e, endocardium.

ing is consistent with a chamber-specific expression of *dHAND* in the developing heart.

dHAND/inv Mutant Embryos Lack a Left-Sided Pulmonary Ventricle

The expression studies of *dHAND* and *eHAND* in wild-type and *inv*/*inv* mice suggest that these factors are chamber-specific rather than side-specific. To further assess this model, *dHAND*-null mice were bred into the *inv*/*inv* genetic background by intercrossing mice heterozygous for both mutations. Just as *dHAND* expression in the *inv*/*inv* embryos was localized to the left side of the embryo, the left-sided ventricle failed to form in the *dHAND*/*inv* null embryos (Fig. 5A). Although the side of the hypoplastic ven-

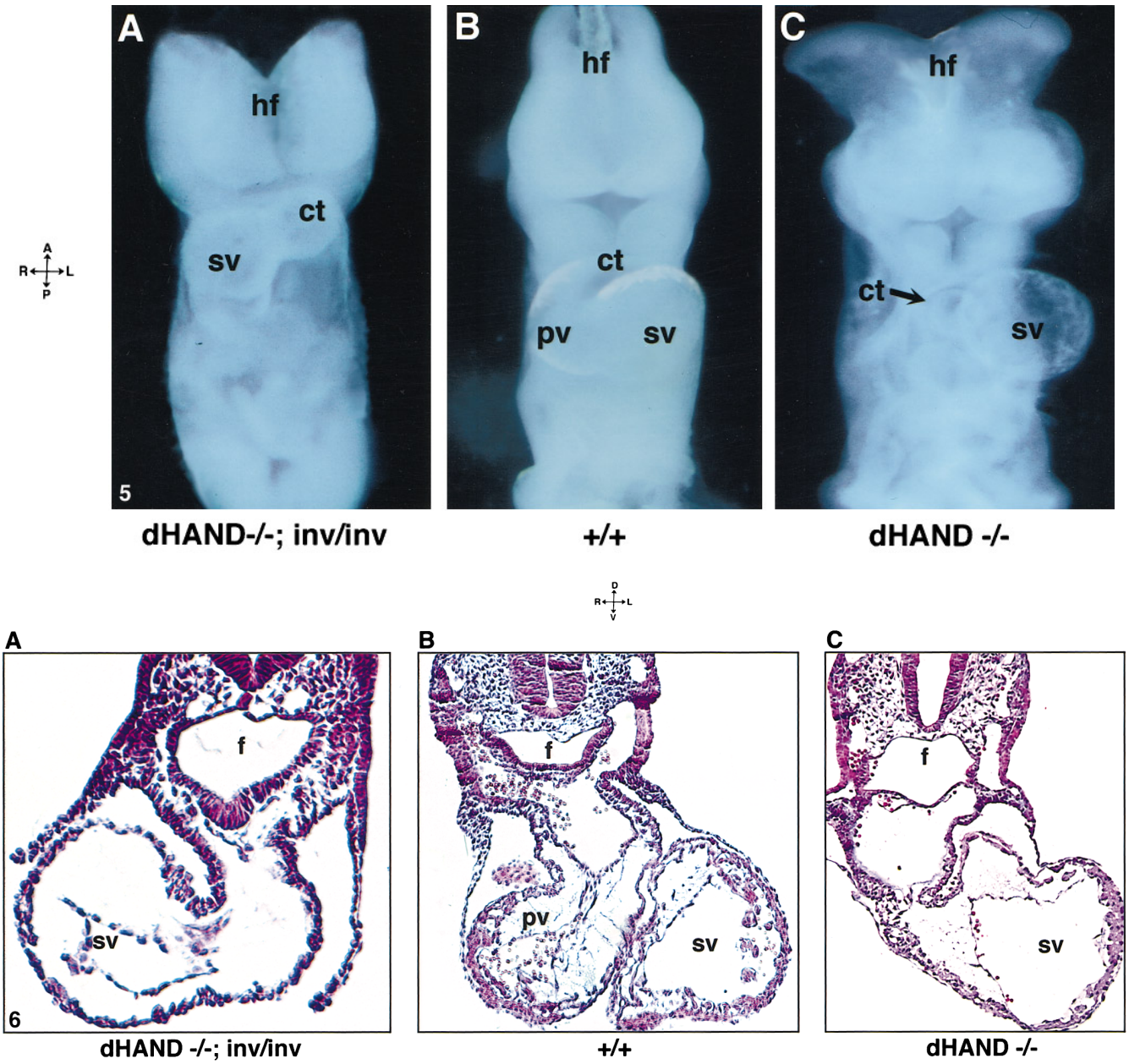

FIG. 5. *dHAND*/*inv* combined mutant phenotype in mouse embryos. Ventral view of an E9.5 embryo homozygous for mutations in *dHAND* and *inv* (A). A single right-sided systemic ventricle (sv) does form; however, the pulmonary ventricle (pv) is hypoplastic. The pv would have been left-sided in the *inv*/*inv* embryo. This is in comparison to a wild-type (+/+) littermate (B) demonstrating a looped heart with regions fated to form the conotruncus (ct), pv (right-sided), and sv (left-sided). In a *dHAND*-null embryo (C), the right-sided pv fails to form, leaving only a left-sided sv. The embryo in A was slightly younger than that in C and dilation of the sv had not yet occurred. hf, head fold.

FIG. 6. Histologic comparison of wild-type, *dHAND*-null, and *dHAND*/*inv* combined mutant mice. Transverse section through the looped heart demonstrates that mice homozygous for *dHAND* and *inv* mutations have a single right-sided ventricle which is systemic (A). Two well-defined ventricular chambers are apparent in wild-type (+/+) mice (B). The pulmonary ventricle (pv) is right-sided and the systemic ventricle (sv) is left-sided. *dHAND*-null hearts have only a left-sided sv (C). Note absence of trabeculations in *dHAND* and *dHAND*/*inv* mutant systemic ventricles. f, foregut.

tricle was reversed in the *inv* background, the pulmonary ventricle was the one affected by the absence of dHAND in both wild-type and *inv* backgrounds (Fig. 5). The remaining ventricle was the *eHAND*-expressing systemic ventricle. Histologic analysis of *dHAND*/*inv* mutant embryos compared to wild-type and *dHAND*-null embryos confirmed the

consistent hypoplasia of a pulmonary ventricle (Fig. 6) in the *dHAND* mutants.

DISCUSSION

Although the two related bHLH transcription factors, dHAND and eHAND, are expressed asymmetrically along the AP and DV axes of the early heart tube, we did not detect LR asymmetry of expression prior to cardiac looping. After cardiac looping, dHAND and eHAND assume a LR cardiac asymmetry which develops only by virtue of morphogenetic movements of the heart tube. Analysis of *HAND* gene expression in situs inversus mice confirmed a chamber-specific pattern of expression. Generation of *dHAND*/*inv* double null embryos reinforced a role for dHAND in formation of the pulmonary ventricle regardless of the anatomic location of the ventricle.

The LR cardiac asymmetry of *eHAND* expression has been interpreted to mean that eHAND lies in a molecular pathway regulating embryonic LR asymmetry (Biben and Harvey, 1997). Asymmetric expression of *shh*, *nodal*, *lefty*, *cSnR-1*, and *activin receptor IIa* along the LR axis occurs well before linear heart tube formation and has been implicated in a molecular cascade controlling the direction of cardiac looping (Levin *et al.*, 1995; Lowe *et al.*, 1996; Collignon *et al.*, 1996; Meno *et al.*, 1996; Isaac *et al.*, 1997). *Nodal*, *lefty*, and *cSnR-1* are expressed asymmetrically in the left or right lateral mesoderm prior to any organ formation, yet initiate a coordinated LR asymmetry of lung, heart, and gut development. There must therefore be downstream mediators of the signaling pathway which would control LR asymmetry within specific organ systems.

The data presented in this paper do not support a role for eHAND as part of the cascade of signals that specifies embryonic LR asymmetry. In contrast to the LR asymmetry of *eHAND* in the linear heart tube shown by Biben and Harvey (1997), we show LR symmetry of *eHAND* expression in the ventral portion of the straight heart tube. How might one resolve this discrepancy? The LR asymmetry described by Biben and Harvey was only seen on histologic analysis. It is possible that the LR asymmetry was very transient in the straight heart tube and that it had become symmetric at the stage shown in this paper. Given the sharp AP border of *eHAND* expression between the future right and left ventricle regions of the straight heart tube, it is also conceivable that a transverse section a few degrees off the perpendicular axis of the midline heart tube would result in a section traversing through the future left ventricle on the left and the future right ventricle on the right. In such a situation, expression of *eHAND* would be apparent on the left side of the heart tube but not the right. The section demonstrating LR symmetry of *eHAND* expression in the straight heart tube shown here (Fig. 2C) suggests that eHAND is not responding to the asymmetric cascade of signaling peptides involved in embryonic LR asymmetry. Rather, the interrupted AP pattern of *eHAND* gene expression is translated to a LR asymmetry by virtue of cardiac looping.

While the right and left ventricles are initially patterned in the AP direction, the left and right atria are not delineated along the AP axis, but rather along the initial LR axis. Defects in the direction of cardiac looping are often associated with atrial isomerism, a condition where both atria are morphologically of the right or left type. Thus, asymmetry of *eHAND* expression in the atrium would suggest a role for eHAND in such LR decisions. However, our data demonstrate symmetrical expression of *eHAND* in the atria, again making it less likely that eHAND is mediating the signaling pathways determining embryonic LR asymmetry.

Although it does not appear that the *HAND* genes play a role in determining the direction of cardiac looping, it remains possible that they may be involved in the process of cardiac looping. The initial DV polarity of expression is later manifested as expression along the outer curvature of the looped heart but not the inner curvature. This was true in wild-type as well as *inv* hearts. It is tempting to speculate that the *HAND* genes may play a role in differential cell proliferation or cell death along the outer curvature compared to the inner curvature thereby regulating the process of looping of the heart tube.

Finally, maintenance of *dHAND* expression in the pulmonary ventricle and *eHAND* in the systemic ventricle regardless of the LR position of the ventricles as seen in wild-type and *inv*/*inv* embryos supports a model where dHAND and eHAND regulate chamber-specific development, rather than laterality decisions. The double mutant (*dHAND*/*inv*) phenotype does not directly address whether eHAND might lie downstream of the *inv* gene. However, the finding of a hypoplastic pulmonary ventricle in *dHAND*/*inv* null mice in spite of LR reversal of the hypoplastic ventricle confirms the chamber-specific role of dHAND. Conceptually, this is a reasonable model given that the pulmonary and systemic ventricles have unique electrical, physiologic, and morphologic properties. Recent analyses of *cis* elements regulating cardiac transcription of myosin light chain 2V (MLC2V) (Ross *et al.*, 1996), myosin light chain 3F (Kelly *et al.*, 1995), SM22 (Li *et al.*, 1996), and desmin (Kuisk *et al.*, 1996) suggest that there are separable regulatory elements controlling expression in the pulmonary and systemic ventricles. dHAND and eHAND provide the first transcriptional bases for such molecular segmentation of the heart.

Congenital heart defects seen clinically support a model of segmental development of the heart. The majority of defects described in newborns affect only a particular chamber or region of the heart rather than affecting the heart globally. In cases where the right ventricle fails to form, the left ventricle is most often normal in every respect; the converse is true if the left ventricle is hypoplastic. These observations are consistent with a molecular model of independent, segmental development of the heart (Fishman and Olson, 1997). Identification of chamber-specific transcription factors such as dHAND and eHAND provides an avenue through which molecular pathways controlling segmental cardiogenesis may be dissected. It will be necessary to determine what the upstream and downstream members of the dHAND/eHAND pathways are and whether muta-

tions in such members are involved in hypoplastic right or left ventricle syndromes.

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