CONTROL OF CARDIAC AND LIMB DEVELOPMENT BY THE bHLH TRANSCRIPTION FACTORS dHAND AND eHAND

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To my mother and father

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and

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by

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Publication No. _____

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Members of the basic helix-loop-helix (bHLH) transcription factor family regulate the specification and differentiation of multiple cell lineages during embryonic development. The bHLH proteins dHAND and eHAND are expressed in complimentary and overlapping patterns during embryogenesis, and gene knockout studies have demonstrated that *dHAND* and *eHAND* null embryos die from defects in right ventricular and placental development, respectively. Therefore, we have investigated three aspects of *HAND* gene biology. Firstly, in order to determine the mechanisms that establish chamber identity during cardiac

development, we have identified a transcriptional enhancer that controls *dHAND* expression in the embryonic right ventricle, and demonstrated that activity of this element depends on paired binding sites for the GATA family of zinc-finger transcription factors. Secondly, we have generated floxed alleles of murine *eHAND* in order to investigate the role of eHAND during heart formation. These studies have identified a novel role for eHAND during cardiac valve formation, and demonstrated genetic redundancy of *HAND* genes during mammalian cardiac morhpogenesis. Finally, we have utilized tissue culture assays and transgenic mice to investigate the mechanisms by which dHAND regulates transcription of downstream target genes. These results suggest that dHAND and eHAND may regulate gene expression independently of direct DNA binding and transcriptional activation.

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* equal contribution

FIGURES

TABLES

ABBREVIATIONS

αMHC	α-myosin heavy chain
A-P	anterior-posterior
ANF	atrial natriuretic factor
AV	atrioventricular
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
cx40	connexin 40
E	embryonic day
Ihh	*Indian hedgehog*
LV	left ventricle
mlc2V	ventricular myosin light chain 2
NF-AT	nuclear factor of activated T cells
OFT	outflow tract
prx1	*paired*-related homeobox 1
RV	right ventricle
Shh	*Sonic hedgehog*
TGFβ	transforming growth factor-β
VEGF	vascular endothelial growth factor
VSD	ventricular septal defect
ZPA	zone of polarizing activity

CHAPTER 1: GENERAL INTRODUCTION

Molecular control of vertebrate heart formation

The vertebrate heart is an excellent model system for studying the complex development of 3-dimensional organs. The complexity involved in generating the human heart is reflected in the frequency of cardiac abnormalities at birth, the highest among all reported birth defects (Hoffman, 1995). Because of the complexity and striking variability of cardiac malformations, it was once believed that the vast majority resulted from environmental insults. However, the recent identification of single gene mutations responsible for complex cardiovascular phenotypes has ushered in a new era of research into the genetic underpinnings of congenital heart disease.

Cells in the anterior lateral plate mesoderm form a crescent-shaped group of cells (referred to as the cardiac crescent) that express genetic markers of the cardiac lineage. Specification of vertebrate cardiac mesoderm occurs concurrently with gastrulation. Bone morphogenetic protein (BMP) signals from the endoderm induce cardiomyocyte fate, whereas Wnt-mediated signals from the underlying neural tube and notochord suppress cardiomyoctye specification. Coordination of these signaling gradients is accomplished by modulating the suppressive Wnt signals; the secreted Wnt-binding proteins, Crescent and Dkk-1, inhibit Wnt activity in the anterior endoderm, and thereby define the heart field in an area of low Wnt activity and high BMP signal strength (Marvin et al., 2001; Schneider and Mercola, 2001; Schultheiss et al., 1997; Tzahor and Lassar, 2001). These extracellular inductive signals are interpreted in the nucleus by transcription factors that then activate the

myocardial gene program. Two classes of transcription factors that have been implicated in myocardial specification are the Nk class of homeodomain proteins and members of the GATA family of zinc finger transcription factors (Bruneau, 2002; McFadden and Olson, 2002).

Subsequent to formation of the cardiac crescent, heart precursors migrate to the ventral midline of the embryo and fuse to form a linear heart tube composed of an outer layer of myocardium and an inner layer of endothelium separated by an extracellular matrix known as the cardiac jelly. The heart tube loops or folds to the right, a process that serves to position the inflow and outflow segments at the anterior pole of the heart, as well as to align the future cardiac chambers. The direction of cardiac looping has been conserved in all vertebrates examined, and is controlled by a signaling pathway that regulates chirality of the internal organs (Capdevila et al., 2000; Kathiriya and Srivastava, 2000).

Cells in discrete regions of the cardiac crescent are fated to contribute to specific chambers or regions of the adult heart (Yutzey and Bader, 1995). This patterning is apparent as early as the linear heart tube stage as reflected by the restricted expression patterns of several cardiac transcription factors. However, morphologic distinction of the different chambers does not occur until after heart looping. Careful examination of the expression patterns of several genes during early heart morphogenesis has provided evidence that a distinct transcriptional program orchestrates formation of chamber myocardium at the outer curvatures of the primitive heart (Christoffels et al., 2000). The inner curvature and outflow tract retain the transcriptional signature and electrophysiological characteristics of the primary heart tube myocardium while chamber myocardium expands, trabeculates, and

develops a high velocity conduction system (Moorman et al., 2000). This "ballooning" of the chamber myocardium occurs after cardiac looping, and genetic markers for this growth are upregulated along the outer curvatures of the heart tube (Christoffels et al., 2000).

Several mouse knockout phenotypes lend support to this hypothesis. Mice lacking the MADS-box transcription factor MEF2C or the basic helix-loop-helix (bHLH) protein dHAND fail to properly develop the right ventricle and outflow tract, and die at embryonic day 9.5-11.5 (E9.5-11.5) (Lin et al., 1997; Srivastava et al., 1997). In addition, *Nkx2.5* null mice die between E9.5 and E11.5, and form a single ventricular chamber that fails to express the basic helix-loop-helix transcription factor *eHAND*, a marker of the left ventricular chamber (Biben and Harvey, 1997; Lyons et al., 1995; Tanaka et al., 1999a). In all cases, formation of the primary heart tube is not notably disturbed, suggesting that these phenotypes represent failures in the transcriptional program that controls expansion of chamber myocardium.

Generation of *Nkx2.5/dHAND* compound null mice results in a severe reduction in ventricular precursors, suggesting that expansion of both the right and left ventricular chamber myocardium is precluded in the double null background (Yamagishi et al., 2001). This suggests that Nkx2.5 controls expansion of the posterior segments of the myocardium (LV), whereas dHAND controls expansion of the anterior segments (RV, outflow). Mice lacking the T-box transcription factor Tbx5 display similar defects in the posterior segment (Bruneau et al., 2001). Interestingly, Nkx2.5 and Tbx5 physically interact and synergistically activate transcription of the *atrial natriuretic factor* (*ANF*) and *connexin 40* promoters,

providing a potential mechanistic explanation for the overlapping phenotypes observed in heterozygous and null embryos (Bruneau et al., 2001; Hiroi et al., 2001).

Concomitant with looping and expansion of the ventricular chambers, the endocardial cushions form and remodel to generate the outflow (aortic and pulmonary) and atrioventricular (mitral and tricuspid) valves, as well as portions of the atrial and ventricular septa. Endocardial cushions arise as swellings of the cardiac jelly that become cellularized as endothelial cells delaminate and undergo an epithelial to mesenchymal transformation. Studies have implicated TGFβ signaling as a positive factor and VEGF signaling as an inhibitory factor for endocardial transformation (Brown et al., 1999; Dor et al., 2001; Nakajima et al., 1994). Two sets of cushions form at specific junctions within the immature heart tube, and myocardium at these sites is uniquely capable of inducing endothelial cell transformation (Eisenberg and Markwald, 1995).

Following delamination and transformation of the endothelium, cells within the cushion mesenchyme proliferate as the cushions enlarge. Studies of mouse mutants have revealed several factors that affect this process, including the transcription factors Sox-4 and NF-ATc, the secreted signaling molecules BMP6 and BMP7, the intracellular docking protein ShcA, and components of the neuregulin signaling pathway (de la Pompa et al., 1998; Gassmann et al., 1995; Kim et al., 2001; Lai and Pawson, 2000; Lee et al., 1995; Meyer and Birchmeier, 1995; Ranger et al., 1998; Schilham et al., 1996). Some of the phenotypes of mice lacking these genes also include a hypoplastic myocardium; therefore, it is unclear whether the cushion defect is secondary to an abnormal myocardium.

After the initial stage of cardiac looping, the mammalian embryonic circulation remains a single, linear system: blood moves from the common atrium into the left ventricular inflow, passes into the right ventricle, then to the outflow tract and systemic circulation. However, as the embryo begins to fashion the serial circulatory system required after birth, significant remodeling of the heart and vascular connections must occur. The atrioventricular cushions and canal must be repositioned to the right to overlie both ventricular inflows. Concurrently, the conal cushions must shift to overlie the right and left ventricles. This is accomplished by remodeling the endocardial cushions and inner curvature of the myocardium, a process involving migration of myocardial cells into the cushion mesenchyme. Defects at this stage can result in hyperplastic cushions and thick valve leaflets as has been noted in *Smad6* and *TGFβ_2* knockout mice (Bartram et al., 2001; Galvin et al., 2000). Concomitant with this remodeling, the primary muscular atrial and ventricular septa grow to meet the AV cushions, completing conversion to the embryonic parallel circulatory system. Defects in the coordination of this remodeling result in common developmental cardiac defects, including double outlet right ventricle and ventricular septal defects, as exemplified in *TGFβ_2* knockout mice (Bartram et al., 2001).

HAND genes and embryonic development

Members of the basic helix-loop-helix family of transcription factors play critical roles in the specification, differentiation, and morphogenesis of multiple tissues and organs during embryonic development (Massari and Murre, 2000). The bHLH proteins dHAND and eHAND are expressed in overlapping and complimentary patterns in cardiac, pharyngeal

arch, and sympathetic neurons during mammalian embryonic development (Cserjesi et al., 1995; Srivastava et al., 1995; Srivastava et al., 1997). Within the heart, *dHAND* is expressed broadly throughout the ventricular and atrial precursors; however, the highest levels are detected within the right ventricle. In contrast, *eHAND* is expressed exclusively in the outer curvatures of the left ventricle and outflow tract (Biben and Harvey, 1997; Srivastava et al., 1997).

Targeted mutations in *dHAND* and *eHAND* have revealed critical roles for each factor during embryonic development. Ablation of *dHAND* in mice results in embryonic lethality around E10.5 as a result of cardiac and vascular defects (Srivastava et al., 1997; Yamagishi et al., 2000). In *dHAND* $^{-/-}$ embryos, the heart forms normally up to stage E8.0; however, expansion of the right ventricular myocardium fails, and thus at later stages appears hypoplastic. One potential explanation for hypoplasia of the right ventricle is an increase in apoptosis that is detected in the right ventricle at E10.5 (Yamagishi et al., 2001). This suggests that dHAND is required for the survival of these precursors. Interestingly, expansion of the left ventricle appears to be unaffected.

In addition to its role during cardiac development, dHAND plays a key role in patterning anteroposterior (A-P) axis of the developing limb. Growth and patterning of the limb bud along the A-P axis is controlled by the zone of polarizing activity (ZPA), a specialized region of mesoderm at the posterior margin of the limb bud. Grafts of the ZPA into the anterior margin of a recipient limb bud result in mirror-image duplications of the distal skeletal elements (Saunders and Gasseling, 1968). The secreted protein Sonic hedgehog (SHH) is localized to the ZPA, and application of beads soaked in recombinant

SHH to the anterior margin of the limb results in mirror-image digit duplications (Riddle et al., 1993). Limbs from *Shh* mutant mice also show severe defects in patterning along the A-P and dorsoventral axes and lack polarizing activity in grafting experiments (Chiang et al., 2001; Chiang et al., 1996).

dHAND is expressed in the posterior limb mesenchyme prior to *Shh*, and its expression pattern completely encompasses that of *Shh* at all later stages of limb development (Charité et al., 2000). Moreover, limb buds from mice and zebrafish lacking dHAND fail to upregulate *Shh* expression (Charité et al., 2000; Yelon et al., 2000). Misexpression of dHAND in the anterior limb mesoderm of mouse or chick embryos is also sufficient to induce ectopic *Shh* and mirror image duplications of posterior skeletal elements (Charite ét al., 2000; Fernandez-Teran et al., 2000).

In contrast to *dHAND* null mice, which survive until E10.5, mice lacking *eHAND* arrest in development at E8.0 as a result of severe extraembryonic defects. Morphologic defects are present in amnion, yolk sac, and trophpblast tissues; in addition, placental lactogen-1, a hormone secreted by trophoblast giant cells, is downregulated in *eHAND* mutants (Firulli et al., 1998; Riley et al., 1998). Markers of cardiac myocyte differentiation are expressed normally in mutant embryos, suggesting that eHAND is not required for cardiac specification or differentiation (Firulli et al., 1998). However, analysis of cardiac morphology is precluded in the context of severe extraembryonic malformations. Tetraploid aggregation experiments have been used to rescue the extraembryonic defects present in *eHAND* null embryos. Rescued embryos arrest in development at E10.5 and display defects in cardiac morphogenesis (Riley et al., 1998). However, is it unclear whether potential

defects in other tissues contribute to this phenotype. In addition, a complete analysis of the cardiac malformations in *eHAND* null embryos has not been described.

In addition to overlapping expression during cardiac, lateral mesoderm, and neural crest development, several additional lines of evidence suggest that dHAND and eHAND may share redundant functions during cardiac development. Antisense experiments in chicken embryos have revealed that inhibition of both dHAND and eHAND was required to interfere with cardiac morphogenesis. Incubation of chick embryos with antisense molecules to either eHAND or dHAND alone did not affect cardiac development, whereas incubation with antisense molecules to both factors resulted in arrested cardiac development following cardiac looping (Srivastava et al., 1995).

In addition, zebrafish and flies possess only a single *HAND* gene, that exhibits greater similarity to *dHAND*. Mutations in the zebrafish *hands off* locus, that encodes dHAND, result in severe defects in cardiac and limb development. *hands off* mutants form a reduced amount of myocardial tissue that fails to express ventricular myosin heavy chain, suggesting that the ventricular compartment of the heart is most severely affected by the loss of HAND function (Yelon et al., 2000). Because this defect is more severe than those noted in mice lacking dHAND, it is possible that *eHAND* expression in the left ventricular compartment compensates for the loss of dHAND. This may explain the relatively normal formation of the left ventricular chamber in *dHAND*$^{-/-}$ embryos. However, the early embryonic lethality observed in *eHAND* null mice precludes an easy test of this hypothesis by generating double knockout embryos.

Transcriptional regulation by HAND proteins

Although gene knockout experiments identify dHAND and eHAND as critical regulators of cardiac, vascular, limb, and extraembryonic development, their mechanism of action as transcription factors remains unclear. In general, tissue-specific (class B) bHLH factors form obligate heterodimers with ubiquitous bHLH proteins (class A), called E-proteins (Massari and Murre, 2000). Dimerization juxtaposes the basic regions of bHLH proteins to form a bipartite DNA binding domain that recognizes the E-box consensus sequence (CANNTG) with resulting transcriptional activation or repression of target genes.

eHAND and dHAND were identified in protein-protein interaction screens using E proteins as baits and have been shown using biochemical techniques to interact and regulate transcription in conjunction with E-proteins (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995). eHAND has been shown to bind a nonconsensus E-box (NNTCTG) sequence as a heterodimer with the E-protein E47 (Hollenberg et al., 1995). Interestingly, reporter gene assays in tissue culture cells have suggested that eHAND may have dual functions as both a transcriptional repressor and activator (Hollenberg et al., 1995; Scott et al., 2000). The protein domain responsible for transcriptional repression mapped to the bHLH region, suggesting that the activity of eHAND and may be regulated, in part, through the choice of bHLH partner.

More recent studies have demonstrated that eHAND and dHAND have promiscuous dimerization selectivity. Both dHAND and eHAND have been shown to homodimerize as well as heterodimerize with each other (Firulli et al., 2000; Scott et al., 2000). In addition, biochemical evidence suggests that HAND proteins may heterodimerize with other bHLH

proteins, such as members of the hairy-related transcription factor (HRT) family (Firulli et al., 2000). Interestingly, E proteins are not expressed in trophoblast giant cells at a time when eHAND is expressed and required for giant cell differentiation (Scott et al., 2000). This provides strong circumstantial evidence that HAND proteins may not act according to traditional models of class B bHLH function in vivo.

Objectives

We have investigated three avenues of HAND transcription factor biology during mouse embryonic development. First, because of the crucial role dHAND plays in right ventricular chamber morphogenesis, we have identified and characterized a 5'-cis acting transcriptional regulatory element that directs *dHAND* transcription in the developing heart. We demonstrate that two paired binding sites for the GATA family of zinc-finger transcription factors are required for activity of this enhancer in vivo, suggesting that dHAND acts downstream of GATA factors during development of the right ventricular chamber.

Second, in order to circumvent the early embryonic lethality observed in *eHAND* null mice, we have generated floxed alleles of *eHAND* by homologous recombination. We have also obtained and generated transgenic mice expressing Cre recombinase in the embryonic heart, and used these to generate cardiac-specific deletions of *eHAND*. Using this approach, we demonstrate that cardiac expression of *eHAND* is not required for viability until after birth. *eHAND* conditional knockout mice die approximately two days after birth, and display ventricular septal defects and thickened atrioventricular valves. These results identify a

novel role for eHAND during endocardial cushion maturation. In addition, reduction of *dHAND* gene dosage by 50% in *eHAND* conditional knockout mice results in embryonic lethality at E10.5, demonstrating genetic redundancy of mammalian *HAND* genes.

Finally, we have investigated the transcriptional properties of dHAND using tissue culture assays and transgenic mice. We demonstrate that dHAND binds DNA and activates transcription as a heterodimer with E proteins in tissue culture. Transcriptional activity of dHAND in these assays requires direct DNA binding and an amino-terminal transcriptional activation domain. In order to determine if dHAND functions by similar transcriptional mechanisms in vivo, we have misexpressed dHAND mutant proteins in the anterior mesoderm of the limb bud, and assayed transgenic animals for the formation of ectopic digits. Surprisingly, dHAND activity in transgenic mice is independent of direct DNA binding and the transcriptional activation domain. These results suggest that dHAND acts via novel mechanisms during limb development, and warrants the investigation of the protein partners that cooperate with dHAND to pattern the developing limb.

CHAPTER 2: A GATA-DEPENDENT RIGHT VENTRICULAR ENHANCER CONTROLS dHAND TRANSCRIPTION IN THE DEVELOPING HEART

Introduction

Classical embryology and cell fate mapping experiments have defined the morphological events involved in formation of the vertebrate heart (Fishman and Chien, 1997; Olson and Srivastava, 1996). Cardiac precursors in the anterior lateral plate mesoderm become committed to a cardiogenic fate in response to signals from the adjacent endoderm at embryonic day 7.5 (E7.5) in the mouse. These cells form the cardiac crescent and give rise to paired bilaterally symmetric cardiac primordia. As development continues and the embryo closes ventrally, the cardiac primordia fuse along the midline at E8.0 to form a single, linear, beating heart tube. Shortly thereafter, the linear heart tube loops to the right, signifying the first morphological manifestation of embryonic left-right asymmetry. Cardiac looping serves to juxtapose the future left and right ventricles, and to align the inflow and outflow tracts with the atrial and ventricular chambers. Following looping, septation of both ventricular and atrial chambers occurs, and populations of migrating neural crest cells contribute to the outflow tract and great vessels as the heart develops and grows to form the mature four-chambered organ (Jiang et al., 2000; Le Lievre and Le Douarin, 1975).

Although the morphologic events of cardiac development are well characterized, the underlying molecular mechanisms that specify different cardiac cell fates and regulate regional-specific patterns of gene expression in the developing heart are only beginning to be revealed. Recent analyses of several cardiac transcription factors and the genetic cascades in

which they act have suggested that heart formation proceeds in a segmental fashion, with each cardiac chamber governed by an individual genetic program (Fishman and Olson, 1997). Interestingly, atrial and ventricular cardiomyocytes, which exhibit differences in gene expression, contractility, morphology, and electrophysiology, appear to be specified well before chamber morphogenesis (Yutzey and Bader, 1995). There are also molecular distinctions between cardiomyocytes in the right and left ventricular chambers, but how these cells acquire their distinctive features is unknown.

Members of the basic helix-loop-helix (bHLH) family of transcriptional regulators play diverse roles in cell specification and differentiation in many tissues (Massari and Murre, 2000). The related bHLH proteins, dHAND and eHAND, also known as Hed/Thing-2 and Hxt/Thing-1, respectively, show dynamic and complementary expression patterns in the developing heart (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995). During mouse embryogenesis, *dHAND* and *eHAND* are expressed in the cardiac crescent concomitant with cardiogenic specification (Biben and Harvey, 1997; Thomas et al., 1998b). As the heart tube forms, *dHAND* is expressed throughout the ventral surface of the linear heart tube and, at the onset of cardiac looping, is downregulated in the future left ventricle (LV), with expression being maintained along the outer curvature of the right ventricle (RV) and in the outflow tract (OFT) (Thomas et al., 1998b). In contrast, *eHAND* is downregulated in the region of the linear heart tube that contributes to the RV and, following looping, is expressed along the outer curvature of the LV and in portions of the OFT. The specific expression patterns of *dHAND* and *eHAND* exemplify the remarkable territoriality of gene

expression in the developing heart and intimate the existence of spatially-restricted transcriptional networks acting at the very earliest stages of cardiogenesis.

Consistent with their chamber-restricted expression patterns, the *HAND* genes appear to be essential for morphogenesis of their respective cardiac chambers. Mice homozygous for a null mutation in *dHAND* fail to form a RV and have severe abnormalities in the neural crest-derived aortic arch arteries, resulting in embryonic demise at E10.0 (Srivastava et al., 1997; Yamagishi et al., 2000). *eHAND* null embryos die from severe extraembryonic defects around E8.0, making a clear analysis of heart development difficult (Firulli et al., 1998; Riley et al., 1998). However, tetraploid rescue experiments, employed to circumvent the early embryonic lethality, have shown that rescued animals arrest at E10.5 with apparent defects in maturation of the left ventricular chamber (Riley et al., 1998). In the zebrafish, which has only a single ventricular chamber, only a single *HAND* gene, most closely related to *dHAND*, has been identified (Angelo et al., 2000; Yelon et al., 2000). Loss-of-function mutations in this gene, called *hands off*, result in ventricular ablation, suggesting that this single gene in the fish shares the combined functions of *dHAND* and *eHAND* in chamber morphogenesis in the mouse (Yelon et al., 2000).

Several other mouse mutants result in regionally-restricted abnormalities in cardiac morphogenesis, further reinforcing the notion of chamber-specific transcriptional programs. Mice lacking the MADS-box transcription factor MEF2C, expressed throughout the heart, exhibit defects in cardiac looping and an absence of a future RV (Lin et al., 1997). This phenotype is highly reminiscent of the *dHAND* mutant phenotype. Whether the absence of a RV in *MEF2C* mutants, and the consequent absence of *dHAND* expression, reflects a role of

MEF2C as a regulator of *dHAND* is unknown. Mice homozygous for a null mutation in the homeobox gene *Nkx2.5* display defects in looping morphogenesis, an apparent absence of the left ventricular chamber (Lyons et al., 1995; Tanaka et al., 1999a), and downregulation of *eHAND* expression, suggesting that *eHAND* is a key downstream target of Nkx2.5 that may contribute to cardiac defects in these animals (Biben and Harvey, 1997).

Analysis of the *cis*-regulatory elements of several cardiac genes in transgenic mice has also demonstrated the existence of anatomically-restricted transcriptional programs that act through independent enhancers to control transcription in different regions of the heart (Firulli and Olson, 1997; Kelly et al., 1999). In many cases, transcription factors that are expressed throughout the heart are required for regionally-restricted activation of cardiac enhancers. How such factors act selectively in specific subsets of cardiomyocytes is unknown.

In the present study, we analyzed the *dHAND* upstream region for regulatory elements sufficient to recapitulate the chamber-restricted expression pattern of the endogenous gene. We describe independent enhancers that direct expression of a *lacZ* transgene in the heart and branchial arch neural crest in patterns that mimic *dHAND* expression throughout development. Deletion analysis of the cardiac-specific enhancer revealed that subregions regulate transcription in the OFT and RV and that two conserved binding sites for GATA transcription factors are required for enhancer activity in the RV. These results identify GATA factors as direct upstream activators of *dHAND* and demonstrate that the transcriptional activity of GATA family is regulated in a chamber-specific manner during heart development.

Results

Identification of *dHAND* cardiac and branchial arch enhancers

In order to define the cis-regulatory elements that control *dHAND* expression during cardiac development, two overlapping phage clones spanning approximately 11 kb of upstream genomic sequence were isolated. To determine if these sequences contained regions important for *dHAND* expression, a fragment from the 5' untranslated region (UTR) to –11 kb was fused to *lacZ* and tested for expression at E10.5 (Fig. 1, construct 1). Transgenic embryos showed X-gal staining in the heart as well as the first and second branchial arches. Expression in the heart at E10.5 was primarily localized to the outer curvature of the future RV and the OFT. Lower levels of expression were detected in the future LV (Fig. 1), a pattern that recapitulates that of endogenous *dHAND* transcripts (Thomas et al., 1998b). Stable transgenic lines harboring construct 1 showed the same expression pattern of *lacZ* at E10.5 as that seen in F_0 transgenics (data not shown). Of note, this 11 kb upstream region did not direct expression in lateral mesoderm, limb buds, or sympathetic ganglia, where *dHAND* is also expressed (Charite et al., 2000; Srivastava et al., 1995). This suggested that the regulatory elements responsible for expression within these tissues are located elsewhere.

To further localize the regulatory elements responsible for cardiac expression of *dHAND*, a fragment extending from the 5'UTR to –5.5 kb was fused to *lacZ* (Fig. 1, construct 2). This construct directed *lacZ* expression only in the developing heart, implying that the branchial arch enhancer lay between –5.5 and –11 kb, whereas the cardiac regulatory region was downstream of –5.5 kb. Subsequent analysis localized the branchial arch

FIGURE 1

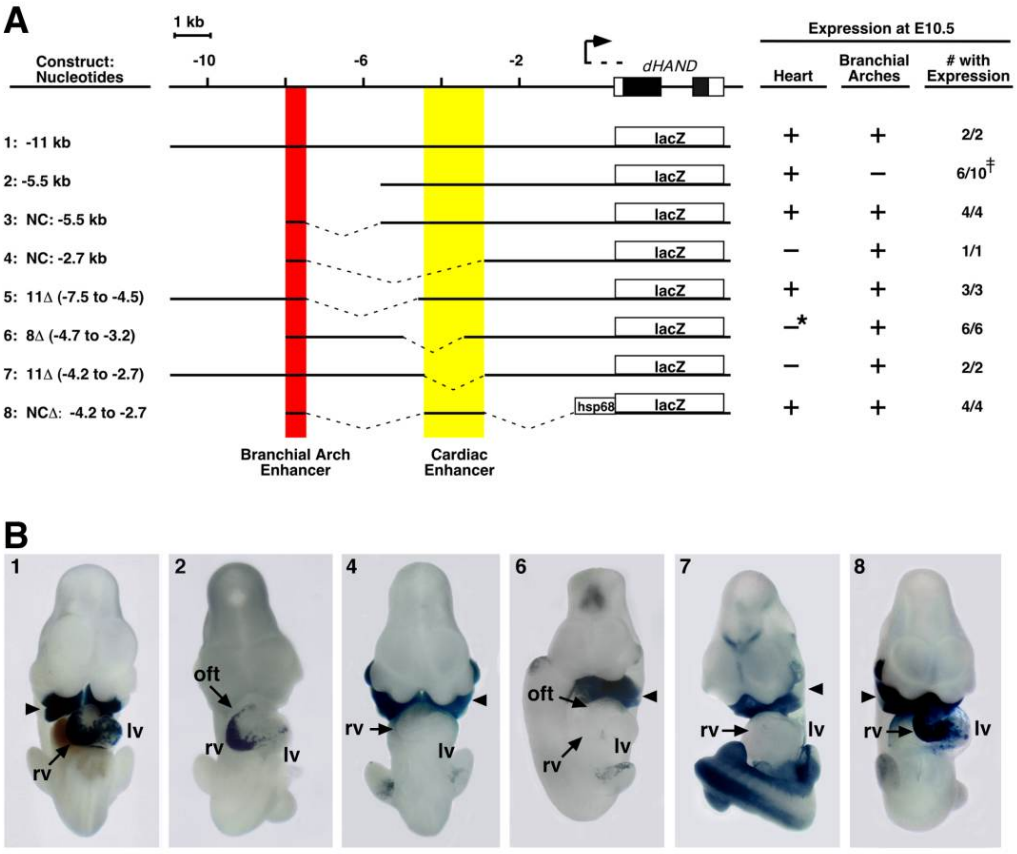


Fig. 1. Transgene constructs used to identify the *dHAND* branchial arch and cardiac enhancers. (A). Shown at the top is the genomic organization of the mouse *dHAND* locus and 5' flanking region. Construct number is indicated at the left, and the corresponding expression pattern is summarized at the right. The far-right column indicates the fraction of F0 transgenic embryos showing heart expression expression that also showed branchial arch expression, except for construct 2. For that construct (indicated by †) , which lacks the branchial arch enhancer, we observed lacZ staining in the hearts of 5 out of 9 stable transgenic lines and in one F0 embryo. (B). Representative embryos obtained with each construct (indicated in upper left corner of each panel) are shown. Embryos with constructs 1 and 2 are from stable transgenic lines, whereas those with constructs 4, 6, 7, and 8 are from F0 transgenics. Constructs 1-7 contained the indicated *dHAND* upstream region linked to the promoterless AUGβgal reporter. Branchial arches are indicated by arrowheads. * Indicates that *lacZ* expression was seen in only a few cells that were always restricted to the right ventricle. rv, right ventricle; lv, left ventricle; oft, outflow tract.

enhancer to the region between -8.0 and -7.75 kb (Charite et al., 2001). The level of cardiac expression from construct 2 was lower than from construct 1, although both showed preferential expression in the right ventricle. Since we found no cardiac regulatory sequences between -5.5 and -11 kb, the higher level of expression directed by the 11 kb region may reflect the existence of a quantitative element in this region that cannot function alone or sensitivity of the shorter region to integration effects.

To facilitate a rapid analysis of the location of regulatory sequences responsible for cardiac expression of *dHAND* and to control for possible integration effects, we included the branchial arch enhancer in all subsequent deletion constructs. First, however, to rule out potential interference between the two regulatory regions, we tested the branchial arch enhancer fused to the 5' end of construct 2 (Fig. 1, construct 3). This construct directed *lacZ* expression in the branchial arches and heart. Staining in the heart was identical to construct 2, leading us to conclude that the branchial arch enhancer did not interfere with the cardiac regulatory region when placed directly upstream (data not shown). Construct 4, which contained the branchial arch enhancer and deleted the region from –7.5 to –2.7 kb abolished cardiac *lacZ* expression. In conjunction with construct 2, this suggested that the cardiac regulatory region was located between –5.5 and –2.7 kb. Deletion of 3 kb from –7.5 to –4.5 kb from the entire 11 kb upstream region did not abolish cardiac expression (Fig. 1 construct 5), suggesting that a cardiac enhancer was between –4.5 kb and –2.7 kb. Construct 6, which contained a deletion from –4.7 to –3.2 kb, abolished all cardiac gene expression, except in a small number of cells always localized to the RV. Deletion of the region between –4.2 and –2.7 kb, in the context of 11 kb of upstream region, abolished all cardiac gene expression (Fig.

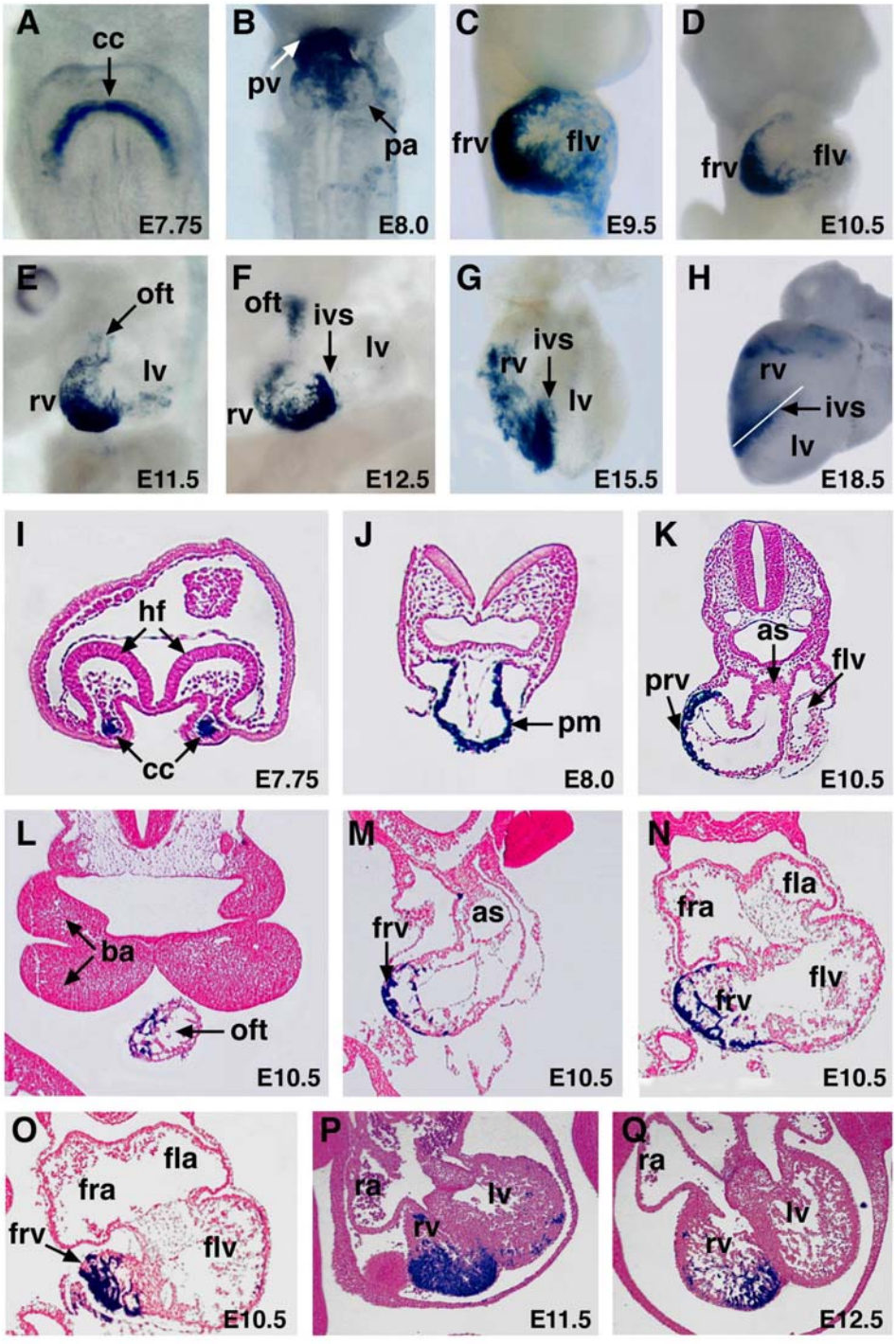
1, construct 7). This demonstrated that sequences in this region were required for cardiac *dHAND* expression. To test whether these elements were sufficient to direct cardiac expression, we fused the region from –4.2 to –2.7 kb and the branchial arch enhancer to the *hsp68* basal promoter upstream of *lacZ* (Fig. 1, construct 8). This construct directed *lacZ* expression in the branchial arches and heart, allowing us to conclude that the cardiac enhancer of *dHAND* lay between –4.2 and –2.7 kb.

Expression of the *dHAND* cardiac enhancer during embryonic development

We next sought to determine whether the *dHAND* cardiac enhancer was sufficient to recapitulate the complete spatiotemporal pattern of expression of endogenous *dHAND*. Therefore, we generated stable transgenic lines with construct 2, containing the 5.5 kb upstream region fused to *lacZ* . Nine transgenic lines were obtained, five of which showed comparable expression patterns during heart development. Line 165 showed robust expression of *lacZ*, and was therefore chosen for further characterization.

At E7.75, line 165 showed *lacZ* expression throughout the cardiac crescent, in a pattern identical to endogenous *dHAND* mRNA (Fig. 2A, I) (Thomas et al., 1998b). At E8.0, lacZ was detected throughout the myocardial layer of the linear heart tube (Fig. 2B, J). At the onset of cardiac looping (E8.25), *lacZ* expression was downregulated in the future LV, mirroring the regulation of endogenous *dHAND* transcripts (data not shown). Following the completion of cardiac looping (after E8.5), *lacZ* expression was detected along the outer curvature of the future RV, OFT, and pericardium, while lower levels were seen in the future LV (data not shown and Fig. 2C, D, K). Because the expression pattern of this enhancer

FIGURE 2


A
cc
E7.75
B
pv
pa
E8.0
C
frv
flv
E9.5
D
frv
flv
E10.5
E
oft
lv
rv
E11.5
F
oft
ivs
lv
rv
E12.5
G
ivs
rv
lv
E15.5
H
rv
ivs
lv
E18.5
I
hf
cc
E7.75
J
pm
E8.0
K
as
flv
prv
E10.5
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O
fla
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flv
E10.5
P
ra
lv
rv
E11.5
Q
ra
lv
rv
E12.5

Figure 2. Expression of the *dHAND* cardiac enhancer throughout embryonic development. Embryos from stable transgenic line 165 harboring construct 2 were analyzed at various times during mouse embryogenesis. (A-H) Whole-mount photographs of embryos or isolated hearts from E7.75-E18.5. The embryo in D is the same as in Fig. 1B2. (I-Q) Nuclear Fast Red counterstained transverse sections. Note strong staining in the cardiac crescent (cc) at E7.75 (A, I). As the linear heart tube forms, expression is detected throughout the primitive myocardium (pm) of the linear heart tube (B, J). Note lack of expression in the primitive atrium (pa) (B). Following cardiac looping at E9.5 (C, K), X-gal staining is detected at high levels primarily in the outer curvature of the future right ventricle (frv), but at low levels, in a patchy pattern, in the future left ventricle (flv). Serial sections of an E10.5 embryo (D, L-O) show strong expression in the outer curvature of the right ventricle and portions of the outflow tract (oft); however, *lacZ* expression is not seen in the atria or aortic sac (as). Lower, patchy levels are again noted in the future left ventricle (flv). Later during development, between E11.5-E18.5, (E-H, P-Q), *lacZ* expression is detected at lower levels, but is still restricted primarily to the right ventricle (rv), especially at the apex and in portions of the interventricluar septum (ivs), and regions of the outflow tract (E-F). Lower levels can be seen at the base of the left ventricle (lv) (E-F). hf, head folds; ba, branchial arches; fra, future right atrium; fla, future left atrium; ra, right atrium.

replicates the dynamic pattern of endogenous *dHAND* mRNA expression during cardiac looping, we believe it responds to the signaling pathways that establish chamber-restricted expression of endogenous *dHAND*.

Following heart looping, the pattern of *lacZ* expression continued to mimic the pattern of endogenous *dHAND* mRNA. At E10.5, the graded right-left ventricular expression of *lacZ* was readily visible (Fig. 2D, L-O). Serial transverse sections of an E10.5 embryo revealed expression along the outer wall of the OFT and RV (Fig. 2L-N), as well as the base of the RV (Fig. 2O). Expression of *lacZ* was never detected in the atrial chambers (Fig. 2N-Q). At later stages of cardiac development, *lacZ* expression was downregulated; however, overnight X-gal staining allowed us to detect low levels of activity. At E11.5-12.5, expression was detected at low levels in the RV and OFT, with the highest levels noted at the base of the RV near the junction of the interventricular septum (IVS) (Fig. 2E, F, P, Q). At E15.5 and E18.5, low levels of *lacZ* expression were detected in the RV (Fig. 2G-H). The highest levels of transgene expression detected at later time points localized to the apex of the RV and IVS (Fig. 2G, H).

Anatomic reversal of *dHAND* enhancer activity in inv/inv mice

The right ventricular expression of *dHAND* is anatomically reversed in *inv/inv* mice, which display a reversal of left-right asymmetry, reflecting the orientation of the pulmonary ventricle to the left and the systemic ventricle to the right (Thomas et al., 1998b). To test whether the *dHAND* cardiac enhancer also responded to the inv pathway in a manner analogous to the endogenous gene, we introduced transgene construct 2 into the *inv/inv*

background by breeding. As shown in Fig. 3A, the enhancer was active on the left side of the heart in *inv/inv* embryos, corresponding to the position of the pulmonary ventricle. We conclude that the *dHAND* cardiac enhancer responds to transcriptional signals specific for the pulmonary ventricle, which normally is on the right, rather than signals for left-right asymmetry per se.

Activity of the *dHAND* cardiac enhancer in mouse mutants with altered ventricular development.

We also introduced transgene construct 2 into several mouse mutants with altered ventricular development as a means of analyzing the fates of *dHAND*-expressing cells in hearts with various dysmorphologies. In *MEF2C* mutant embryos, the right ventricular region fails to develop (Lin et al., 1997). As shown in Fig. 3B, *dHAND-lacZ* expression was localized to the conotruncal region of *MEF2C* mutant hearts at E9.5, with almost no staining in the more posterior region of the heart, confirming that right ventricular cells that would normally express *dHAND* are absent in this mutant.

In *dHAND* mutant embryos, *dHAND-lacZ* showed an expression pattern essentially identical to that in *MEF2C* mutants (Fig. 3C), consistent with the conclusion that the right ventricular chamber is missing in these embryos (Srivastava et al., 1997). Expression of *lacZ* was maintained in the conotruncal region, demonstrating that these cells do not require dHAND for specification or survival. The expression of *dHAND-lacZ* in these cells of the mutant also suggests that dHAND does not positively autoregulate its expression in these cells.

FIGURE 3

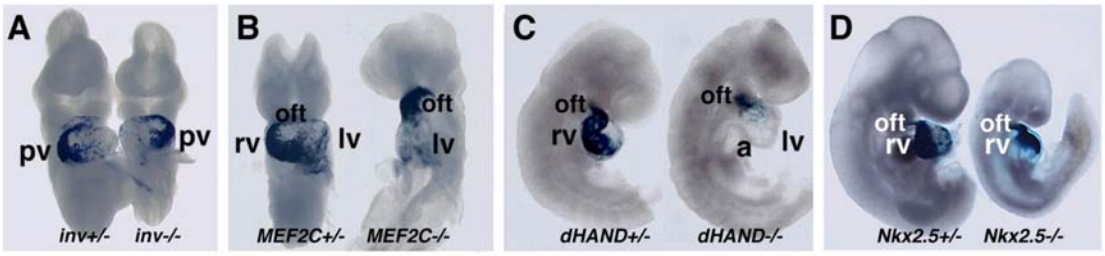


Figure 3. Altered expression of the *dHAND* cardiac enhancer in mice with aberrant ventricular development. Construct 2 (Fig. 1) was introduced by breeding into the indicated mutant backgrounds and embryos were stained for *lacZ* expression. (A) Frontal views of $inv^{+/-}$ and $inv^{-/-}$ embryos at E9.5. In homozygous *inv* mutants, *lacZ* expression is reversed from the right to the left side of the heart, marking the position of the developing pulmonary ventricle (pv). (B) Frontal views of $MEF2C^{+/-}$ and $MEF2C^{-/-}$ embryos at E9.5, showing the loss of *lacZ* expression from the right ventricular (rv) region, but maintained expression in the outflow tract (oft). (C) Lateral views of $dHAND^{+/-}$ and $dHAND^{-/-}$ embryos at E9.5. These mutant embryos show similar distortions in *lacZ* expression to those seen in $MEF2C^{-/-}$ embryos. (D) Lateral views of $Nkx2.5^{+/-}$ and $Nkx2.5^{-/-}$ embryos at E10.0. LacZ expression extends into the right ventricular region of the mutant heart, which is morphologically distorted.

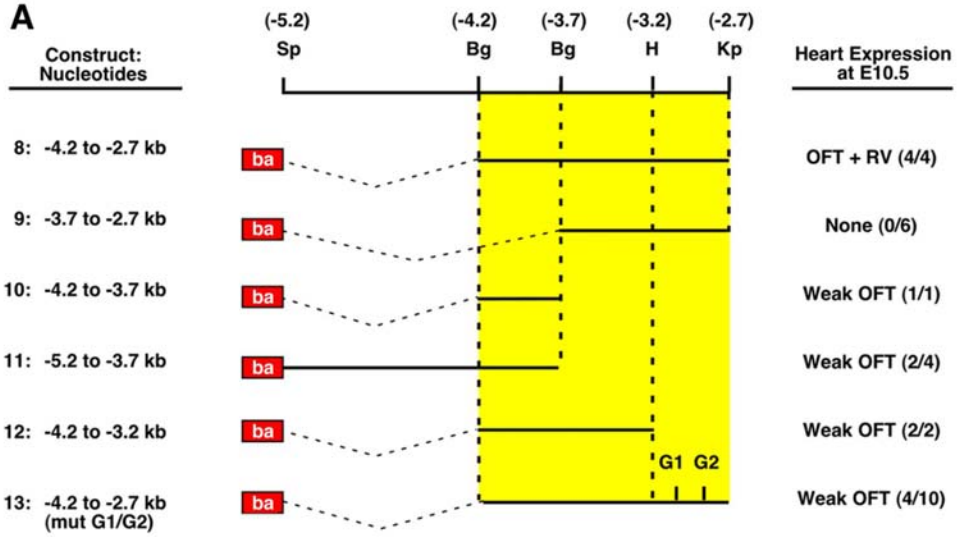
In *Nkx2.5* mutant embryos, *dHAND-lacZ* expression extended from the conotruncus into the more posterior region of the heart tube (Fig. 3D). We interpret this to indicate that the right ventricular region is present in these embryos, but defects in looping and possibly in left ventricular development (Biben and Harvey, 1997) result in an altered distribution of lacZ-positive cells in the remainder of the heart.

Region-specific elements within the *dHAND* cardiac enhancer.

To more precisely define the regulatory elements within the 1.5 kb enhancer, we created a series of deletions of this fragment, and analyzed their transcriptional activity in F_0 embryos using the *hsp68-lacZ* reporter and the *dHAND* branchial arch enhancer as an internal control for integration effects. As mentioned above, the region from –4.2 to –2.7 kb (construct 8) was sufficient to direct expression of *lacZ* in the developing heart (Fig. 4B). Deletion of –4.2 to –3.7 kb completely abolished cardiac expression, suggesting that the enhancer was located in this region (Fig. 4A, C, construct 9). However, this 500 bp alone, or with an additional 1 kb of upstream sequence, was nearly inactive, directing only low level expression in a few cells of the distal OFT (data not shown and Fig. 4A, D, constructs 10 and 11).

Based on these data, it was possible that we split a critical element at –3.7 kb. To test this possibility, we fused the region from –4.2 to –3.2 kb to the *hsp68-lacZ* reporter (construct 12). Interestingly, this construct directed expression in the OFT, but not the right ventricle (Fig. 4E). This suggested that separable elements were present in the 1.5 kb enhancer which controlled expression in different regions of the developing heart. In

FIGURE 4


A
Construct: Nucleotides
(-5.2) Sp
(-4.2) Bg
(-3.7) Bg
(-3.2) H
(-2.7) Kp
Heart Expression at E10.5
8: -4.2 to -2.7 kb
9: -3.7 to -2.7 kb
10: -4.2 to -3.7 kb
11: -5.2 to -3.7 kb
12: -4.2 to -3.2 kb
13: -4.2 to -2.7 kb (mut G1/G2)
ba
G1 G2
OFT + RV (4/4)
None (0/6)
Weak OFT (1/1)
Weak OFT (2/4)
Weak OFT (2/2)
Weak OFT (4/10)

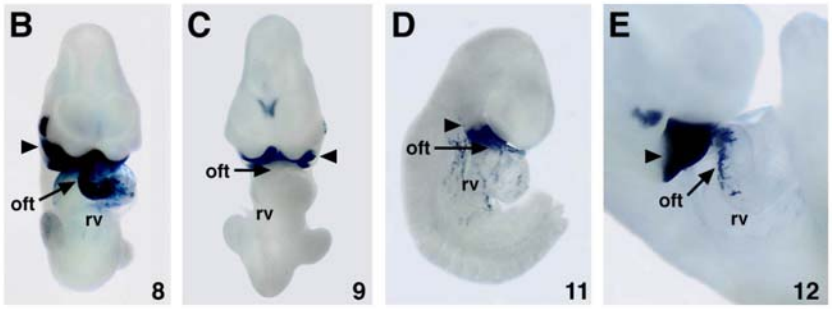

B
oft
rv
8
C
oft
rv
9
D
oft
rv
11
E
oft
rv
12

Figure 4. Fine mapping of chamber-specific enhancer elements. Deletions of the 1.5 kb *dHAND* cardiac enhancer (contained in construct 8) were generated and fused to the *hsp68lacZ* reporter downstream of the *dHAND* branchial arch (ba) enhancer. Construct number and fragment location are indicated at the left, and the *lacZ* expression pattern at E10.5 is summarized to the right. Numbers of embryos with the indicated cardiac expression patterns out of all embryos showing branchial arch staining are indicated in parentheses. (A) Map of the 1.5 kb enhancer and reporter constructs. (B). Staining pattern of embryo harboring construct 8. (C) Construct 9 contained the region from –3.7 to –2.7 kb, and showed no cardiac gene expression in transgenic embryos. Construct 10, containing the region from –4.2 to –3.7 kb, and construct 11, containing the region from –5.2 to -3.7 kb, showed identical patterns of expression, with *lacZ* detected in a few cells of the outflow tract (oft) (D). Construct 12 contained the region from –4.2 to –3.2 kb, and directed expression in the OFT (E). Construct 13, derived from construct 8, contained mutations in two GATA binding sites (indicated as G1 and G2) and directed weak expression in the oft (see Fig. 7B). Note strong X-gal staining in the branchial arches (arrowhead). rv, right ventricle; oft, outflow tract.

addition, because this deletion abolished RV expression, ventricular expression must require sequences in both the 5' and 3' regions of the 1.5 kb fragment. This is consistent with the expression pattern of construct 6 (Fig. 1), in which the region from –4.7 to –3.2 kb was deleted. In this context, most cardiac *lacZ* expression was abolished; however, a few cells, always located in the RV, stained with X-gal. This implies that elements downstream of –3.2 kb have some activity in cells of the RV.

Conservation of *dHAND* regulatory elements

To guide us in identifying potential regulatory elements within the *dHAND* cardiac enhancer, we isolated a human *dHAND* genomic phage clone and compared the upstream sequences of mouse and human *dHAND*. This alignment revealed a high degree of homology to the corresponding mouse sequence throughout the upstream region. The degree of homology was not significantly different within the putative cardiac enhancer compared to flanking regions (data not shown). Alignment of the 1500 bp enhancer, which is both required and sufficient for ventricular expression, revealed conservation of several potential transcription factor binding sites (Fig. 5 and data not shown). In particular, two consensus GATA factor binding sites at –3039 (G2) and –3140 (G1) bp were completely conserved in mouse and human sequences.

FIGURE 5

Figure 5. Alignment of the *dHAND* 5' flanking region from mouse and human
Bestfit alignment of the region from –4.2 to –3.7 kb revealed conservation of several potential transcription factor binding sites. Two consensus GATA factor binding sites are indicated in blue. Restriction sites shown in Fig. 4 are highlighted in yellow.

GATA-dependent regulation of *dHAND* transcription

To determine if the conserved GATA sites were capable of binding GATA4 in vitro, we performed electrophoretic mobility shift assays (EMSA) using oligonucleotide probes. COS-1 cells were transfected with a GATA-4 expression vector, and whole cell extracts were used in the assay. As shown in Fig. 6, GATA-4 specifically shifted the wild-type probe containing the GATA site at –3140 bp, and DNA binding was effectively competed by an excess of unlabeled cognate competitor. However, the unlabeled mutant site was unable to compete for GATA-4 binding. Similar results were seen with oligonucleotide probes containing both the –3039 and –3140 GATA sites.

To determine if these GATA sites were required for transgene expression *in vivo*, we mutated these sites in the context of the 1.5 kb fragment (-4.2 to –2.7 kb) and generated F_0 embryos with this construct (Fig. 4A, construct 13; Fig. 7B). In 10 embryos expressing *lacZ* in the branchial arches at varying levels, cardiac expression was dramatically reduced, compared to the wild-type construct (Fig. 7, compare A (wild-type) and B (mutant)). As expected from our deletion analyses (Fig. 4, constructs 10 and 11), which showed OFT expression with constructs extending upstream of -3.7 kb and lacking the above GATA sites, residual expression was detected in the OFT with construct 13 (Fig. 7B).

To test whether GATA-4 was uniquely required for enhancer activity *in vivo*, we crossed the 5.5 kb enhancer transgenic line (construct 2) into a *GATA-4* mutant background (Molkentin et al., 1997). Analysis of *lacZ* expression in *GATA-4* null embryos showed that transgene expression was maintained in the absence of GATA-4 (Fig. 7C) *GATA-4* mutant embryos exhibit cardia bifida due to a defect in ventral morphogenesis of the embryo (Kuo et

al., 1997; Molkentin et al., 1997). Intriguingly, *dHAND-lacZ* expression was much stronger in the right compared to the left cardiac tube in the mutant, suggesting that the mechanisms that restrict *dHAND* expression to the right ventricular chamber at the onset of cardiac looping are at least partially intact in these embryos. The finding that the GATA-dependent *dHAND* enhancer was active in *GATA-4* mutant embryos suggests that GATA-5 or -6, which are expressed in an overlapping pattern with GATA-4 during cardiac development, can substitute for GATA-4 to activate the *dHAND* enhancer.

FIGURE 6

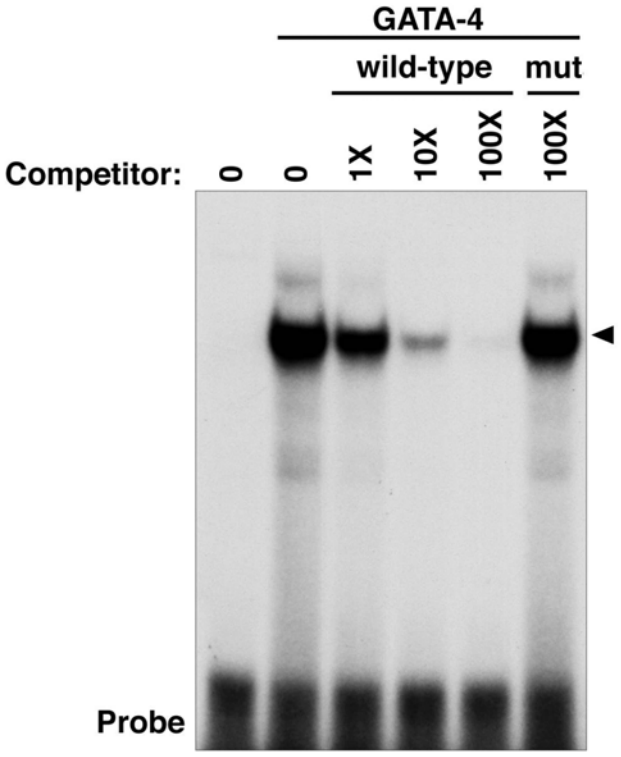


Figure 6. Binding of GATA-4 to the *dHAND* cardiac enhancer in vitro. A ^{32}P-labeled oligonucleotide containing the conserved GATA site at –3140 bp (G1) was used for electrophoretic mobility shift assays with extracts from COS-1 cells. DNA binding was seen only in reactions containing extract from GATA-4-transfected cells. In addition, unlabeled wild-type oligonucleotide efficiently competed for DNA binding, whereas unlabeled mutant oligonucleotide did not compete.

FIGURE 7

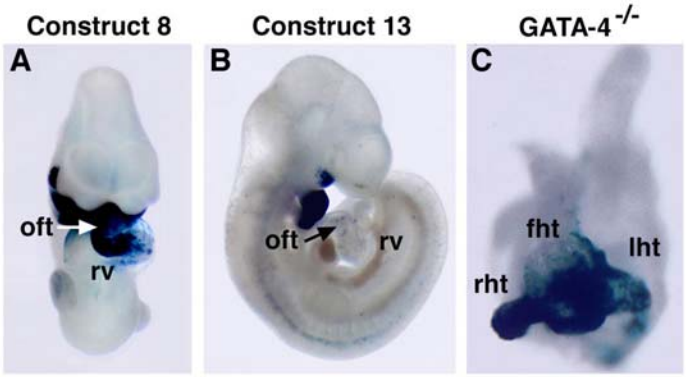


Figure 7. Requirement of GATA sites for activity of the *dHAND* cardiac enhancer in vivo. F_0 transgenic embryos generated with construct 13 containing mutations of the GATA binding sites at –3039 (G2) and –3140 (G1) (see Fig. 4A). (A) E10.5 embryo generated with construct 8 (See Fig. 4A) shows strong expression of *lacZ* in the right ventricle (rv) and outflow tract (oft). (B) Representative embryo harboring construct 13 with mutations in GATA sites G1 and G2 (See Fig. 4A). Note dramatically reduced *lacZ* expression in the heart, with low levels of expression detected primarily in the OFT. (C) Strong expression of construct 2 (see Fig. 1) in both right heart tube (rht), left heart tube (lht), and fused heart tube (fht) of *GATA-4* mutant embryo. Consistent with the expression pattern in wild-type embryos, *lacZ* expression is stronger on the right compared to the left side in the mutant

Discussion

dHAND is an early marker of the future right ventricle and is required for normal development of this cardiac chamber (Srivastava et al., 1997; Thomas et al., 1998b). Thus, identification of the factors acting upstream of *dHAND* should provide insight into the mechanisms for establishment of chamber-specific gene expression during cardiac development. Our results identify an enhancer responsible for early cardiac expression of *dHAND* and show that it is a direct target for activation by GATA transcription factors.

Enhancer modules for distinct regions of the developing heart

Cardiac expression of *dHAND* is controlled by a 1.5 kb enhancer (between –2.7 and –4.2 kb) that encompasses subregions responsible for transcription in the RV and OFT. Deletion of the 3' 500 bp of the enhancer abolished ventricular expression and diminished, but did not eliminate, expression in the OFT. Although the region from –2.7 to –3.2 kb is necessary for transcription in the RV, it is not sufficient for expression in this cardiac segment, and relies on additional sequences within the OFT enhancer, demonstrating the requirement for overlapping, but distinct cis-regulatory regions for *dHAND* transcription in these cardiac segments. We have made numerous attempts to further delineate the boundaries of these enhancers, but all further deletions have abolished all expression, suggesting that essential elements within these enhancers are dispersed over a relatively large region. In contrast, the enhancer responsible for transcription of *dHAND* in the branchial arch neural crest is localized to a region of only about 200 bp, located between -8 and -7.8 kb (Charite et al., 2001).

The modular regulation of *dHAND* transcription in the developing heart is consistent with several recent studies of other cardiac genes and argues against the existence of a general cardiac gene regulatory program (Firulli and Olson, 1997; Kelly et al., 1999). Instead, it appears more likely that cardiac transcription is controlled by unique combinations of cardiac-restricted and widely expressed transcription factors within each cardiac segment.

Transcriptional regulation of *dHAND* by GATA factors

The *dHAND* cardiac enhancer contains two conserved consensus binding sites for GATA factors, which bind GATA-4 with high affinity and are required for enhancer activity. GATA-4/5/6 are among the earliest markers of the cardiac lineage, and are expressed throughout cardiac development (Arceci et al., 1993; Jiang and Evans, 1996; Laverriere et al., 1994; Morrisey et al., 1996; Morrisey et al., 1997). GATA factors, which bind DNA through a zinc-finger motif and act as transcriptional activators (Ko and Engel, 1993; Merika and Orkin, 1993; Morrisey et al., 1997) have been shown to regulate multiple cardiac genes (Charron and Nemer, 1999). Experiments in P19 embryonic carcinoma cells have also shown that inhibition of GATA-4 expression blocks cardiac muscle differentiation, and that overexpression of GATA-4 enhances cardiogenesis (Grepin et al., 1997; Grepin et al., 1995). *GATA-4* null embryos die around E8.5 from a defect in ventral closure, resulting in impaired fusion of the paired cardiac tubes (Kuo et al., 1997; Molkentin et al., 1997). Interestingly, *GATA-6* is upregulated in *GATA-4* null embryos, suggesting that these factors may act redundantly. This may explain the finding that the *dHAND* cardiac enhancer was active in *GATA-4* null embryos.

The phenotype of *dHAND* and *MEF2C* mutant embryos is remarkably similar, with the absence of a right ventricular chamber and defects in looping morphogenesis (Lin et al., 1997; Srivastava et al., 1997). In *MEF2C* mutants, expression of the *dHAND* cardiac enhancer is restricted to the conotruncus, raising the possiblitity that MEF2C is required for *dHAND* expression in the future RV and that in the absence of MEF2C, the dHAND-dependent pathway for RV morphogenesis is not activated. However, there does not appear to be a conserved high-affinity binding site for MEF2 in the *dHAND* cardiac enhancer, which would require an indirect mechanism for possible regulation of *dHAND* by MEF2C. In this regard, recent studies showed that MEF2 factors can be recruited to cardiac genes lacking MEF2 sites, by association with GATA factors (Morin et al., 2000). Thus, it is conceivable that MEF2C might regulate cardiac expression of *dHAND* via the essential GATA sites in the *dHAND* cardiac enhancer.

GATA factors have also been shown to regulate *Nkx2.5* transcription in the cardiac crescent and right ventricle through two independent upstream enhancers (Lien et al., 1999; Reecy et al., 1999; Searcy et al., 1998; Tanaka et al., 1999b). Similarly, the *CARP* promoter contains an essential GATA binding site essential for transcription in a portion of the RV and OFT (Kuo et al., 1999). GATA-dependent transcriptional activation through this site is dependent on Nkx2.5 in vivo and in vitro. However, there are no apparent Nkx2.5 binding sites in the promoter, demonstrating how transcriptional synergy can occur between GATA factors and Nkx2.5 in the absence of cognate sites for both factors. Nkx2.5 also regulates transcription of *GATA-6* in the RV (Davis et al., 2000; Molkentin et al., 2000). Thus, Nkx2.5 and GATA transcription factors participate in mutually reinforcing regulatory networks in

the developing heart in which each factor can activate and maintain the expression of the other.

Potential mechanisms for chamber-restricted expression of *dHAND*

Although GATA factors are required for *dHAND* enhancer activity in the right ventricular chamber, there is no apparent chamber-restriction of GATA factor expression in the developing heart. This suggests that *dHAND* expression requires additional factors, which cooperate with GATA factors to control right-ventricular transcription.

In principle, the highly restricted expression pattern of *dHAND* in the right ventricular chamber (and *eHAND* in the left) could be controlled by chamber-specific transactivators or by negative regulators that suppress expression of the genes in the opposite chambers. While we cannot rule out the latter possibility, our deletion analyses failed to reveal evidence for negative regulatory elements that, when deleted, result in ectopic expression of *dHAND* in the left ventricle, leading us to favor a model in which chamber-restricted coregulators cooperate with GATA factors to confer right ventricular-restricted expression to *dHAND*.

As described above, GATA-4 has been shown to cooperate with Nkx2.5 and MEF2 to activate cardiac target genes (Durocher et al., 1997; Morin et al., 2000; Sepulveda et al., 1998). Transcriptional activity of GATA factors has also been shown to be inhibited by the multitype zinc-finger protein FOG-2, expressed predominantly in the heart (Lu et al., 1999b; Svensson et al., 1999; Tevosian et al., 1999). However, none of these factors show selective expression in one ventricle or the other. One possibility is that chamber-specific signals may be interpreted by cardiac transcription factors that themselves do not show chamber-

specificity of expression, but which activate cardiac target genes only in the presence of appropriate signals. In this regard, the transcription factor NFAT3, which is activated by specific calcium-dependent signals, can cooperate with GATA factors to activate transcription (Molkentin et al., 1998), and repression of GATA-dependent transcription by FOG-2 can be relieved by certain signaling pathways (our unpublished results). Possible activation of such pathways in specific segments of the heart, either by regionally-restricted ligands or in response to hemodynamic or contractile differences along the looping heart tube, could provide a signal for the highly specific expression patterns of cardiac genes.

Analysis of the *cis*-acting regulatory elements within cardiac enhancers is a powerful tool for decoding the combinatorial interactions regulating chamber-specific gene expression during heart morphogenesis. A key issue for the future is to determine how broadly expressed transcription factors cooperate with restricted factors to execute the individual developmental programs each cardiac chamber.

Methods

Cloning and generation of reporter constructs

Two overlapping *dHAND* phage clones were isolated from a mouse 129SV genomic library using the *dHAND* cDNA as a probe (Srivastava et al., 1997). The longest clone contained 11 kb of upstream flanking sequence. Clones were characterized by endonuclease restriction mapping. To generate constructs 1-7, the indicated regions of upstream sequence were fused to the promoterless AUG- gal reporter.

One human *dHAND* genomic clone was isolated from a -phage library and characterized by restriction mapping and sequence analysis. Sequences were aligned with the mouse genomic flanking sequence using the Bestfit algorithm of GCG software.
To generate constructs 8-12, a 750 bp fragment containing the *dHAND* branchial arch enhancer (Charite et al., 2001) was blunt-cloned into the *Hind*III site of the hsp68lacZ vector (Kothary et al., 1989). The orientation of the branchial arch enhancer was confirmed by restriction mapping and DNA sequence analysis. The resulting vector (NC-hsplacZ) was digested with *Sma*I. DNA fragments were amplified from construct 2 by PCR using *Pfu* Turbo (Stratagene) DNA polymerase. The following PCR primer pairs were used: construct 8: Primer 1, 5'-GATCTGTATTTCCCCTTAAAAAGAGACTG-3' and Primer 2, 5'-GAGGAGGGATCCGTACCCTGAATCAG-3.' Construct 9: Primer 3, 5'-GAGGAGGGATCCGATCTATGGTGCTTG-3', and Primer 2. Construct 11: Primer 4, 5'-CTAGTGCTTCATTCTTCCATAAGTCTCAGG-3', and Primer 5, 5'-

TCTGTGATGACGCTGGTTGTGGGC-3'. Construct 12: Primer 1 and Primer 6, 5'-CTTCATGGATCTTAGGAACTCAAACCC-3'.

Construct 10 was generated by *Bgl*II digestion of construct 2, and ligation into NC(Rev)-hsplacZ, which is identical to NC-hsplacZ, except the branchial arch enhancer is in the reverse orientation. All constructs were confirmed by restriction mapping and DNA sequence analysis.

Generation of Transgenic Mice

Reporter constructs were digested with *SalI* to remove vector sequences, and purified for injection using a QiaQuick spin column protocol (QIAGEN). Concentrated DNA was eluted into 10mM Tris buffer, pH7.4. Fertilized eggs from B6C3F1 female mice were collected for pronuclear injection as described (Lien et al., 1999). Foster mother ICR mice were sacrificed at E10.5, and embryos were collected and stained for -galactosidase activity as described (Cheng et al., 1993). Following overnight staining, embryos were fixed for 12 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. For sections, embryos were dehydrated in 2, 2-dimethoxypropane (DMP), cleared in light mineral oil, embedded in paraffin, sectioned at 10 m, rehydrated and stained with Nuclear Fast Red (Moller and Moller, 1994).

Mouse mutants

Mice with mutations in *dHAND* (Srivastava et al., 1997), *MEF2C* (Lin et al., 1997), *Nkx2.5* (Lyons et al., 1995), *inv* (Yokoyama et al., 1993) and *GATA-4* (Molkentin et al., 1997) have been described previously. Inbred C57/BL6 mice were used for intercrosses with *dHAND-lacZ* transgenic mice. Genotypes of embryos were determined by Southern blot analysis of yolk sac DNA.

Electrophoretic mobility shift assays

Oligonucleotides corresponding to the conserved GATA binding sites at –3039 and –3140 were synthesized (Integrated DNA Technologies) as follows:

-3039A, 5'-TCGAGGTGAGGATGCTATCTGGAGCT-3';

-3039B, 5'-TCGAGAGCTCCAGATAGCATCCTCAC-3';

-3140A, 5'-TCGAGGTAATTAACTGATAATGGTGC-3';

-3140B, 5'-TCGAGGCACCATTATCAGTTAATTAC-3'.

Mutant oligonucleotides were also synthesized as follows:

mut3140A, 5'- TCGAGGTAATTAACCCGGGATG-GTGC-3';

mut3140B, 5'-TCGAGGCACCATCCCGGGTTAATTAC-3';

mut3039A, 5'-TCGAGGTGAGGATGGGCCCTGGAGCT-3';

mut3039B, 5'-TCGAGAGCTCCAGG-GCCCATCCTCAC-3'.

Oligonucleotide pairs were resuspended, boiled for 10 min, and annealed by slowly cooling to room temperature. Annealed oligonucleotides were radiolabeled with ^{32}P-dCTP using Klenow fragment of DNA polymerase and purified using G25 spin columns (Roche). COS-1

cells were transfected with pcDNA1-GATA-4 (Lu et al., 1999a) or empty vector. Whole cell extracts were isolated as previously described (Brockman et al., 1995). DNA binding assays were performed as described (Lu et al., 1999a). Unlabeled wild-type and mutant competitor oligonucleotide was added to reactions at the indicated concentrations. DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel in 0.5 X TBE.

Site-directed mutagenesis

Conserved GATA sites at –3039 and –3140 were mutated to *ApaI* and *SmaI* sites, respectively, as follows. DNA was amplified from construct 2 using Primer 1 and Primer 2 (see above), and subcloned into plasmid BK-pBSK for mutagenesis reactions. Two rounds of PCR-based mutagenesis on BK-pBSK were performed using *Pfu* Turbo DNA ploymerase and a QuickChange protocol (Stratagene). The following primers were used: GATAmut3140A, 5'-CCCCTGGATTGTAGTAATTAACCCGGGATGGTGCTG-TCAGTG-3'; GATAmut3140B, 5'-CACTGACAGCACCATCCCGGGTTAATTACTA-CAATCCAGGGG-3'; GATAmut3039A, 5'-CACTTGTGAGGATGGGCCCTGGAGC-TACGAAGCC-3'; GATAmut3039B, 5'-GGCTTCGTAGCTCCAGGGCCCATCCTCA-CAAGTG-3'.

Restriction analysis confirmed the presence of the mutations, and the insert from GATAmutBK-pBSK was again amplified using Primer 1 and Primer 2. This fragment was cloned into the *Sma*I site of NC-hsplacZ. Integrity of the plasmid was confirmed by restriction mapping and sequence analysis.

CHAPTER 3: REGULATION OF MOUSE CARDIAC MORPHOGENESIS BY eHAND AND dHAND

Introduction

Cardiac abnormalities are the most frequent form of human birth defects, and result from aberrant development of the embryonic heart. The molecular pathways that regulate morphogenesis of the multi-chambered vertebrate heart are beginning to be identified (McFadden and Olson, 2002; Srivastava, 2001; Stainier, 2001). Inhibition of negatively-acting Wnt signals in the anterior endoderm allows positively-acting BMP signals to activate the myocardial gene program in a cluster of cells known as the cardiac crescent (Marvin et al., 2001; Schneider and Mercola, 2001; Schultheiss et al., 1997; Tzahor and Lassar, 2001). These cells proliferate and migrate to the midline to form the linear heart tube which subsequently loops to the right (McFadden and Olson, 2002; Stainier, 2001). Subsequently, regions of myocardium along the outer curvatures of the ventricular myocardium expand, trabeculate, and develop a high-velocity conduction system (Christoffels et al., 2000). These regions will give rise to the mature cardiac chambers, whereas myocardium derived from the inner curvature maintains the more primitive genetic signature and conduction system of the primary linear heart tube myocardium (Moorman et al., 2000).

Development of the atrial and ventricular septa, as well as the cardiac valves is coordinated with chamber expansion. Endocardial cushions form at the atrioventricular (AV) and outflow junctions of the early heart when myocardium at these sites secretes a thickened extracellular matrix known as the cardiac jelly (Eisenberg and Markwald, 1995). Endocardium overlying the cardiac jelly undergoes an epithelial to mesenchymal

transformation and invades the matrix, thereby forming the endocardial cushions. The endocardial cushions subsequently remodel into the cardiac valves and septa via processes that are incompletely understood. Abnormalities in morphogenesis of the endocardial cushions result in common forms of human congenital heart disease, including valve abnormalities and septation defects (Bamforth et al., 2001; Eisenberg and Markwald, 1995).

Several classes of transcription factors have been implicated in cardiac morphogenesis and gene regulation (Bruneau, 2002; Firulli and Thattaliyath, 2002). dHAND and eHAND are basic helix-loop-helix transcription factors that are expressed in complimentary and overlapping patterns in the embryonic heart. In mice, *dHAND* is expressed at highest levels in the right ventricle, and at lower levels throughout the atrial and ventricular chambers (Thomas et al., 1998b, and our unpublished observations). In contrast, *eHAND* is expressed exclusively in the outer curvature of the left ventricle and outflow tract (Biben and Harvey, 1997; Thomas et al., 1998b). Targeted mutation of *dHAND* in mice results in lethality at embryonic day 10.5 (E10.5) as a result of right ventricular hypoplasia and vascular malformations (Srivastava et al., 1997; Yamagishi et al., 2000). In contrast, mice lacking eHAND die at E8-8.5 due to severe placental and extraembryonic defects, making analysis of cardiac morphology difficult (Firulli et al., 1998; Riley et al., 1998). However, tetraploid aggregation experiments have been used to circumvent the extraembryonic defects observed in *eHAND* null embryos. Rescued embryos survive until E10.5, and die from cardiac insufficiency as a result of abnormal cardiac looping (Riley et al., 1998). This suggests that eHAND is required for early cardiac morphogenesis. However, the potential requirement of eHAND in other tissues may contribute to embryonic

lethality; in addition, the variable contribution of *eHAND* null cells to the extraembryonic tissues in aggregation experiments may further complicate interpretation of this phenotype.

Experiments in chick and zebrafish suggest that dHAND and eHAND act redundantly during cardiac development. Zebrafish appear to encode only a single *HAND* gene, and mutations in the zebrafish *hands off* locus, that encodes a dHAND ortholog, result in a dramatic reduction in ventricular precursors and (Yelon et al., 2000). This phenotype is more severe than that observed in *dHAND* knockout mice, and suggests that mammalian dHAND and eHAND may act redundantly. In addition, antisense RNA experiments in chick also suggest that both dHAND and eHAND are required for heart morphogenesis, although the phenotype observed in these experiments was much less severe than that of *hands off* mutant zebrafish (Srivastava et al., 1995).

In order to circumvent early embryonic lethality observed in *eHAND* knockout embryos, we have generated floxed alleles of *eHAND* and crossed these to mice expressing cardiac-specific *Cre* transgenes. Surprisingly, despite elimination of *eHAND* transcripts prior to E9.5, these animals survive until birth. Mice lacking myocardial expression of *eHAND* subsequently die within 3 days of birth, and display a variety of congenital heart defects, including ventricular septal defects, double-outlet right ventricle, and hyperplastic atrioventricular valves. In addition, when crossed into the *dHAND*$^{+/-}$ background, *eHAND* conditional knockout mice die at E10.5 and fail to express *atrial natriuretic factor* (ANF) and *connexin 40*. These results identify novel functions of eHAND in ventricular septation and atrioventricular valve development, and demonstrate genetic redundancy between mammalian *HAND* genes.

Results

Generation of floxed *eHAND* alleles

In order to circumvent the early embryonic lethality observed in *eHAND* null mice, we flanked the first exon of *eHAND* with loxP sites by homologous recombination in embryonic stem cells (Fig. 8A). Chimeric male mice generated from ES cells carrying the correctly targeted floxed *eHAND* allele transmitted the mutant allele through the germline. Our targeting strategy included insertion of the neomycin resistance cassette into the 5'-untranslated region of *eHAND*, therefore we suspected that this allele ($eHAND^{NEO\text{-}loxP}$) would be a functional null. Heterozygous $eHAND^{NEO\text{-}loxP}$ mice were viable and fertile; however homozygous $eHAND^{NEO\text{-}loxP/NEO\text{-}loxP}$ embryos were phenotypically indistinguishable at embryonic day 8.0 (E8.0) from our previously reported *eHAND lacZ* knockin mice ($eHAND^{lacZ/lacZ}$) (data not shown and Firulli et al., 1998). This suggested that expression from the targeted locus was abrogated by insertion of the neomycin resistance cassette.

In order to remove the neomycin resistance cassette, we intercrossed heterozygous $eHAND^{NEO\text{-}loxP/+}$ mice to mice expressing the FLPe recombinase in the male germline (Rodriguez et al., 2000). F_1 progeny from these matings were genotyped by Southern blotting to detect the recombined allele ($eHAND^{loxP}$) (Fig 8B). In order to determine if the $eHAND^{loxP}$ allele resulted in hypomorphic expression, we generated $eHAND^{lacZ/loxP}$ adult mice, which were overtly normal and fertile, suggesting that expression from the $eHAND^{loxP}$ allele was not significantly reduced. In addition, $eHAND^{loxP/loxP}$ homozygous mice were

FIGURE 8

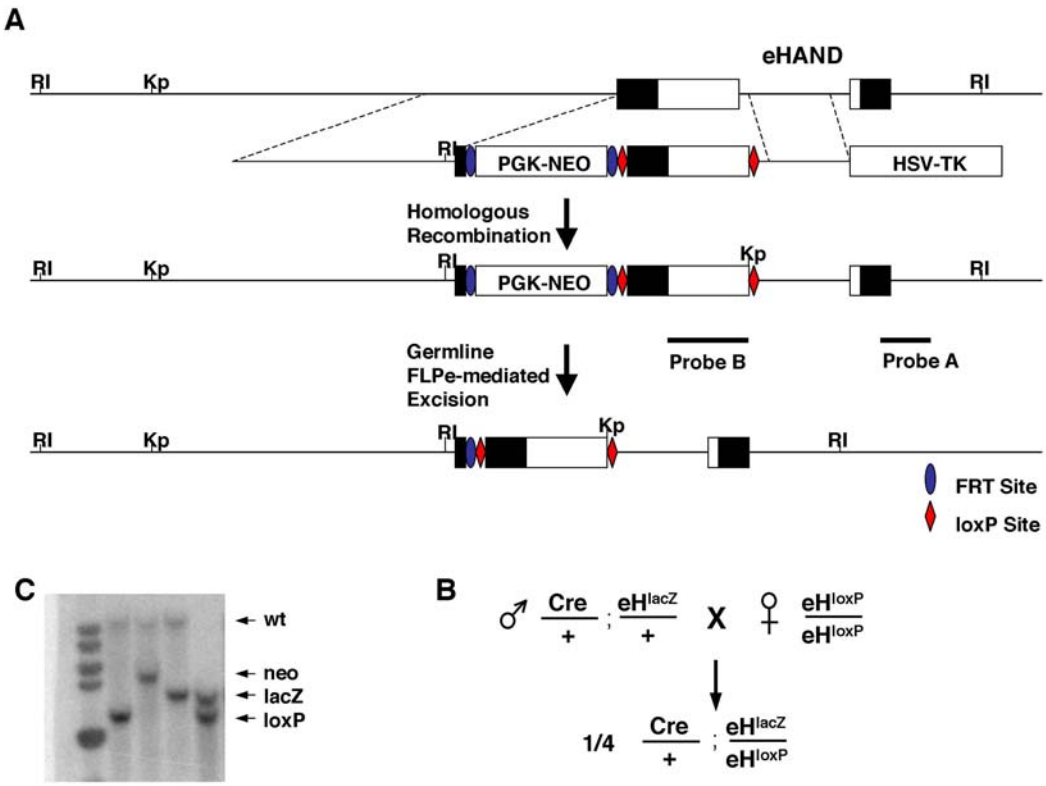


Figure 8. Generation of floxed *eHAND* alleles. (A) LoxP sites were inserted into the 5'-UTR and intron to flank the first exon of *eHAND*. The neomycin resistance cassette was removed in the mouse germline by breeding heterozygous mice to *hACTB::FLPe* transgenic mice. (B) Southern blot analysis using the 3' probe detects all four *eHAND* alleles. (C) Breeding strategy used to generate tissue-specific knockout mice.

phenotypically normal and fertile, and were used in subsequent breedings as diagramed in Figure 8C.

Congenital heart defects in *eHAND*$^{loxP/lacZ}$; *aMHC::Cre* mice

In order to determine the consequences of loss of eHAND in the embryonic heart, we crossed *eHAND*$^{loxP/loxP}$ females to *eHAND*$^{lacZ/+}$ heterozygous male mice harboring the *αMHC::Cre* transgene, that expresses *Cre* under the control of the transcriptional regulatory elements of the *α-myosin heavy chain gene* (*αMHC*). These mice have been reported to express *Cre* in the embryonic and adult myocardium (Agah et al., 1997; Gaussin et al., 2002). Interestingly, several newborn pups from these litters became cyanotic and died within 3 days after birth (P3). Genotyping revealed that these animals were conditional knockout mice (*αMHC-Cre; eHAND*$^{loxP/lacZ}$). This suggested that loss of eHAND in the embryonic myocardium resulted in perinatal lethality. In support of this notion, *αMHC::Cre; eHAND*$^{loxP/lacZ}$ mice were severely underrepresented by P10, and subsequent breedings demonstrated that *αMHC::Cre; eHAND*$^{loxP/lacZ}$ mice were represented at Mendelian frequencies at birth (Table 1 and data not shown).

Histologic sections of P1-2 hearts isolated from wild-type and mutant mice revealed a spectrum of congenital heart defects in *αMHC::Cre; eHAND*$^{loxP/lacZ}$ mice (Fig 9, compare A, B with C-E). Defects frequently observed in *eHAND* mutants included membranous ventricular septal defects (Fig. 9C), overriding aorta (Fig. 9C), double outlet right ventricle (Fig. 9E), and hyperplastic atrioventricular valves (Fig. 9D).

TABLE 1

Genotype	Ratio; Percent
loxP/lacZ	56/181 ; 31%
loxP/lacZ; Cre	7/181 ; 4%
loxP/ +	66/181 ; 37%
loxP/ +; Cre	51/181 ; 28%

Table 1. Observed ratios of genotypes in *eHAND* mutant intercrosses

FIGURE 9

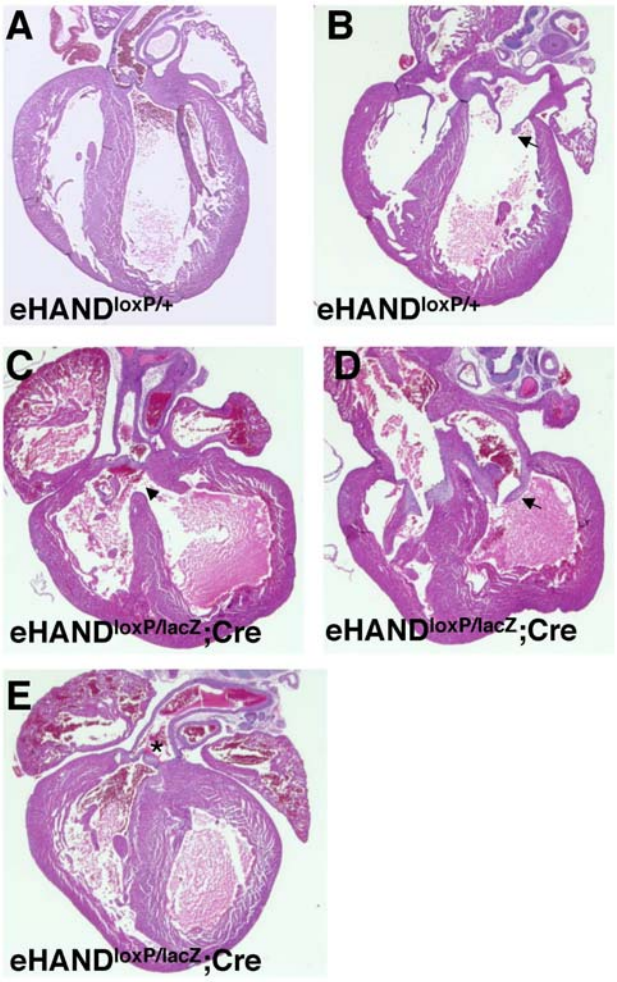


Figure 9. Histology of neonatal mutant hearts. (A) Heterozygous (het) heart demonstrating normal morphology. All animals display membranous ventricular septal defects (VSDs) and overriding aorta. (B) Heterozygous heart showing normal AV valve morphology (black arrow) (C) Mutant heart with membranous VSD (black arrowhead) and overriding aorta. (D) *eHAND* mutant heart with thickened atrioventricular valves (black arrow). (E) Mutant heart with double-outlet right ventricle (asterisk).

Early embryonic Cre-mediated recombination in *αMHC::Cre* mice

Because congenital heart defects result from aberrant embryonic cardiac morphogenesis, and *eHAND* expression is highest during early (E8-E12) embryonic heart development, we suspected that these phenotypes resulted from loss of eHAND in the early embryonic heart. As such, it was important to precisely define the onset of Cre-mediated recombination. Therefore, we intercrossed *αMHC::Cre* transgenics to *ROSA26R* indicator mice (Soriano, 1999), and stained embryos for lacZ expression from E8.0-E11.5. We detected β-galactosidase (β-gal) activity from the *ROSA26* locus as early as E8.0, and by E9.5 recombination was detected throughout the embryonic myocardium (Fig. 10A,B). At E11.5, both atrial and ventricular myocardium expressed high levels of *lacZ*, demonstrating that the *αMHC* regulatory elements direct high level *Cre* expression throughout the early embryonic myocardium (Fig 10C).

In order to demonstrate that the $eHAND^{loxP}$ allele was efficiently recombined in vivo, we analyzed *eHAND* expression in $\alpha MHC::Cre;\ eHAND^{loxP/lacZ}$ embryos at E10.5 by whole mount in situ hybridization. As shown in Figure 10D, *eHAND* transcripts were specifically absent from the embryonic heart, whereas expression of *eHAND* was not affected in the branchial arches and lateral mesoderm. In addition, RT-PCR demonstrated that the majority of transcripts were removed from the LV by E9.5 (Fig. 10E). Cumulatively, these results suggested that efficient cardiac-specific removal of *eHAND* transcripts occurred before E9.5.

FIGURE 10

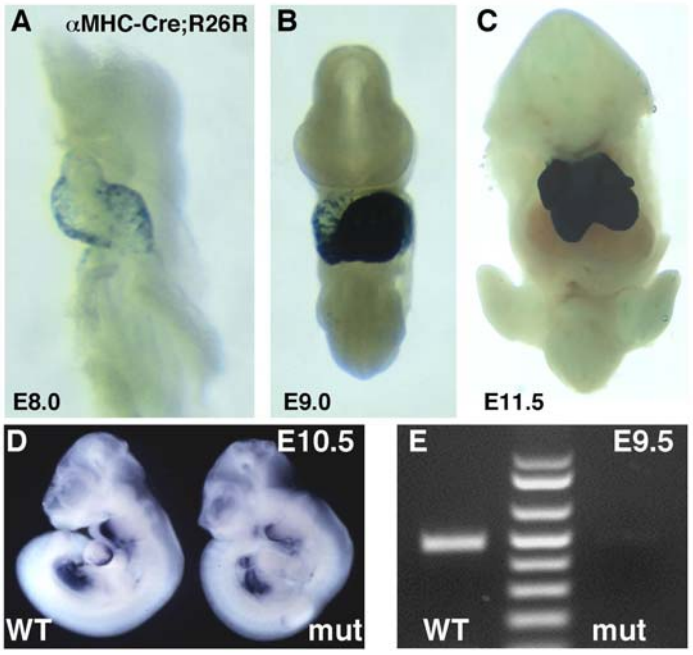


Figure 10. Cardiac-specific Cre-mediated recombination. (A-C) *αMHC::Cre* transgenic mice were intercrossed to *ROSA26R* indicator mice to determine the temporal and tissue specificity of recombination. Whole mount photographs of X-gal stained embryos. (A) E8.0 embryo (B) E9.0 embryo (C) E11.5 embryo (D) Whole mount in situ hybridization to *eHAND* transcripts at E10.5 in wild-type (WT) and mutant (mut) embryos. (E) RT-PCR for *eHAND* transcripts from E9.5 left ventricular total RNA.

Aberrant embryonic heart morphogenesis in *αMHC::Cre; eHAND*$^{loxP/lacZ}$ mice

In order to determine the embryonic consequences of loss of eHAND in the early myocardium, as well as to define the onset of malformations observed at birth, we harvested embryos from timed matings and analyzed morphology by histologic sectioning (Fig. 11). Abnormalities in ventricular septum formation were noted as early as E10.5, and were obvious by E11.5 (data not shown, and Fig. 11A, B). Interestingly, at all stages of development, the muscular ventricular septum appeared thickened and disorganized as compared to wild-type (Fig. 11C,D,G,H,J, compare to A,B,E,F,I). This suggests that the membranous VSD detected at birth is not solely due to atrioventricular (AV) endocardial cushion defects also present in *eHAND* mutants (see below). Interestingly, the left ventricular chamber was reduced in size throughout early embryogenesis (Fig. 11D,G,H); however, at later stages of development and after birth, chamber size appeared to partially recover (Fig. 11J, Fig. 9).

In addition, as early as E11.5, the AV endocardial cushions were hyperplastic relative to wild-type littermates (Fig. 11D,H,J compare to B,F,I). This suggested that an increased number of endocardial cells underwent an epithelial-mesenchymal transformation, or abnormalities in cushion proliferation and remodeling occurred at stages following the epithelial-mesenchymal transformation (see below).

FIGURE 11

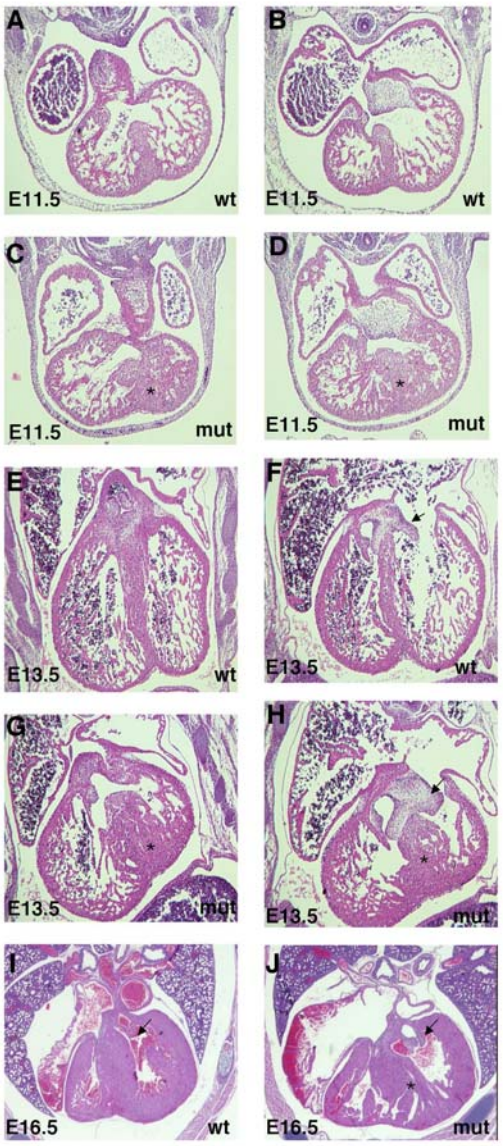


Figure 4. Embryonic histology of *eHAND* mutant hearts. (A,B) Wild-type hearts. (C,D) *eHAND* mutant hearts show a poorly organized ventricular septum (asterisk) and reduced left ventricular size relative to the right ventricle. (E-G) E13.5 embryos (E,F) WT hearts. (G,H) *eHAND* mutants display an incomplete, disorganized ventricular septum, reduced left ventricular dimensions, and immature endocardial cushions (arrow). (I) Wild-type E16.5 heart showing normal AV valves (arrow). (J) Mutant heart showing hyperplastic AV valves (arrow).

Dysregulation of ventricular gene expression

We next sought to identify potential downstream targets of eHAND that may be dysregulated in *αMHC::Cre; eHAND*$^{loxP/lacZ}$ embryos. Several known genes are expressed specifically along the outer curvature of the embryonic ventricles, a pattern partially overlapping with that of *eHAND*. Upregulation of these factors is thought to represent the expansion of the chamber myocardium from the more primitive linear heart tube myocardium (Christoffels et al., 2000). To determine if expansion of the left ventricle was defective in *eHAND* mutants, we examined expression of *connexin 40* (*cx40*), *connexin 43*(*cx43*), *serca2A*, and *Tbx5*. Expression of *cx40* was slightly reduced in the hearts of *eHAND* mutants, however, expression of *cx43*, *serca2A*, and *Tbx5* was similar to wild-type (Fig. 12A,B,F, and data not shown). This suggests that expansion of the left ventricle is not perturbed in *eHAND* mutant hearts.

The transcriptional coactivator cited1 is expressed in a nearly perfectly overlapping pattern with eHAND in the developing heart (Dunwoodie et al., 1998), and mice lacking the closely related factor *cited2* display congenital heart malformations similar to those we observe in *eHAND* mutants (Bamforth et al., 2001). Interestingly, expression of *cited1* was significantly downregulated in *eHAND* mutant hearts (Fig. 12C-E). Expression of *cited2*, which is more broad than that of *cited1* during embryogenesis, was similar in wild-type and mutant hearts (data not shown).

Nkx2.5 null embryos die at E10.5 from left ventricular defects, and fail to express *eHAND* in the heart (Biben and Harvey, 1997; Lyons et al., 1995; Tanaka et al., 1999a). eHAND has therefore been hypothesized to be an important downstream effector of Nkx2.5

FIGURE 12

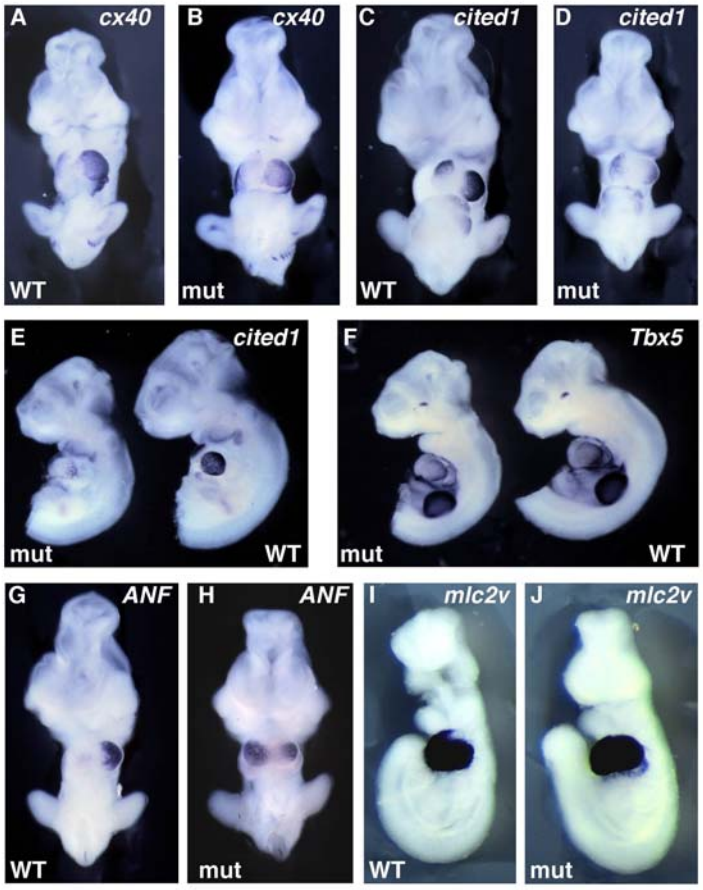


Figure 12. Gene expression analysis in *eHAND* mutant embryos. Whole mount in situ hybridization performed on E10.5 embryos. (A-B) *Connexin 40* (*cx40*) expression in wild-type (A) and mutant (B) embryos. (C-D) Expression of *Cited1* in wild-type (C) and mutant (D) embryos. (E) WT and mutant embryos showing reduction of *Cited1* expression in the LV. (F) WT and mutant embryos showing normal expression of *Tbx5* in *eHAND* mutant embryos. (G-H) *ANF* expression in WT (G) and *eHAND* mutant (H) embryos. (K-J) Expression of *mlc2v* in WT (K) and mutant (J) embryos.

during cardiac morphogenesis. Several genes have been shown to be downregulated in the hearts of *Nkx2.5* null mice; therefore, to determine if Nkx2.5 may regulate these genes indirectly through eHAND, we examined expression of Nkx2.5 target genes by whole mount or section in situ hybridization.

Atrial natriuretic factor (*ANF*) is expressed in the embryonic left ventricle in a pattern similar to eHAND, and is absent in *Nkx2.5* null hearts (Tanaka et al., 1999a). Therefore, we examined *ANF* expression by whole mount and section in situ hybridization. Interestingly, expression of *ANF* in the left ventricle was slightly reduced in *eHAND* mutant hearts; however, expression of *ANF* was dramatically upregulated in the right ventricle of *eHAND* mutants (Fig. 12H, compare to G). In addition, *ventricular myosin light chain 2* (*mlc2v*) has been reported to be downregulated or absent in *Nkx2.5* null hearts (Lyons et al., 1995; Tanaka et al., 1999a). However, we detected no change in *mlc2V* expression in *αMHC::Cre; eHAND*$^{loxP/lacZ}$ embryos (Fig. 12I,J). Together these results suggest that Nkx2.5 regulates transcription of *ANF* and *mlc2v* independently of eHAND.

Endocardial cushion defects in *eHAND* mutant hearts

Unexpectedly, histologic analysis of embryonic and neonatal hearts revealed thickened atrioventricular valves and hyperplastic endocardial cushions. Cardiovascular defects in mice lacking *Smad6* and *TGFβ*$_2$ include thickened cardiac valves (Bartram et al., 2001; Galvin et al., 2000), therefore we examined expression of *Smad6* and *TGFβ*$_2$ in *eHAND* mutants by section in situ hybridization. However, expression of both *Smad6* and *TGFβ*$_2$

FIGURE 13

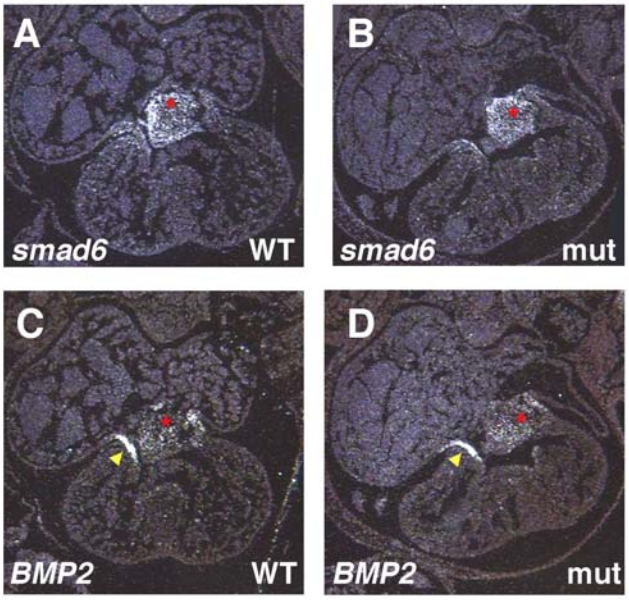


Figure 13. Markers of endocardial cushion morphogenesis in *eHAND* mutant hearts Section in situ hybridization on transverse sections through E11.5 hearts. (A-B) Expression of *Smad6* in wild-type (A) and mutant (B) embryos. Note high level expression in the mesenchmye of the endocardial cushions in both wild-type and mutants (red asterisk). (C-D) Expression of *BMP2* in wild-type (C) and mutant (D) embryos. Note high level expression in the inner curvature of the myocardium (yellow arrowhead) and lower level expression in the mesenchyme of the endocardial cushions (red asterisk).

was normal in mutant hearts at E11.5 and E13.5 (Fig. 13A,B and data not shown). This suggested that dysregulation of other pathways involved in endocardial cushion formation may be responsible for the cushion defects observed in *eHAND* mutant hearts. Therefore, we examined expression of several additional molecules implicated in endocardial cushion differentiation. However, *BMP2*, *BMP4*, and *Smad7* were expressed at normal levels in *eHAND* mutant hearts at E11.5 and E13.5 (Fig. 13C,D and data not shown)

Alterations in cell death and proliferation are known to contribute to endocardial cushion abnormalities observed in *TGFβ_2* and *BMP6/BMP7* mutant mice (Bartram et al., 2001; Kim et al., 2001). Therefore, in order to determine if altered cellular proliferation or apoptosis was responsible for endocardial cushion defects observed in *eHAND* mutants, we examined proliferation and apoptosis in histologic sections. Although slightly more apoptotic cells were present in *αMHC::Cre; eHAND$^{loxP/lacZ}$* hearts at E13.5 (Fig. 14D, compare to C), no differences were observed at earlier stages (Fig. 14A,B). In addition, analysis of cell proliferation at E11.5 by staining with α-phospho histone H3 antibody failed to reveal differences in the number of proliferating cells (data not shown). These results suggest that dysregulated cell proliferation or death after E10.5 is not responsible for the endocardial cushion defects observed in *αMHC::Cre; eHAND$^{loxP/lacZ}$* embryos.

Generation of Nkx2.5::Cre mice

Because the *αMHC::Cre* transgene directs high levels of *Cre* expression after E8.0, it is possible that eHAND is required for cardiac morphogenesis prior to and during this stage of

FIGURE 14

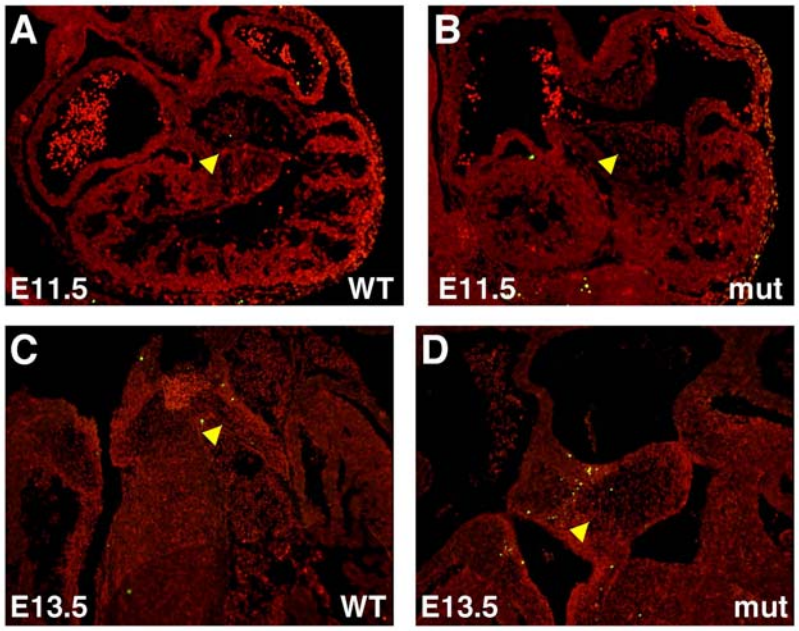


Figure 14. Apoptosis in *eHAND* mutant hearts. (A-B) TUNEL assay performed on E10.5 embryo sections in wild-type (A) and mutant (B) hearts. Apoptotic cells are detected with fluorescein-conjugated antibody. Note similar, low numbers of apoptotic cells in both wild-type and mutants. (C-D) TUNEL assay performed on E13.5 embryo sections through the AV canal. Note slightly increased apoptosis in the endocardial cushion of *eHAND* mutant embryos (D).

development. Therefore, the postnatal phenotype we observe in *αMHC::Cre; eHAND*$^{loxP/lacZ}$ mice may represent an incomplete phenotype due to elimination of eHAND transcripts during, but not before, the time window during which it is required. To address this possibility, we generated a nuclear localization signal (NLS)-*Cre* expression cassette under the control of the transcriptional regulatory sequences of *Nkx2.5* (Fig 15A). These sequences direct expression at the onset of cardiac commitment, as *Nkx2.5* is among the earliest markers of the myocardial fate (Lien et al., 1999). In addition, these sequences direct transgene expression in the thyroid primordium, and portions of the pharynx, regions where *eHAND* is not expressed.

We generated six *Nkx2.5::Cre* transgenic lines, and crossed three into the *ROSA26R* heterozygous background to examine the efficiency and tissue-specificity of Cre-mediated recombination. All three lines exhibited a similar pattern of β-gal activity, which included heart, pharynx, and a subset of cells within the liver (data not shown). However, line 9 directed the highest levels of recombination in the heart, and was therefore used for all subsequent experiments. We detected efficient recombination within the myocardium of *Nkx2.5::Cre* transgenic mice at the linear heart tube stage, and recombination occurred throughout the heart tube by E8.5 (Fig. 15B,C). Serial sections through X-gal stained E8.5 embryos revealed that the majority of cells in the right and left ventricular myocardium underwent recombination. Lower efficiency of recombination was also detected in the endocardium and endocardial cushions (Fig. 15D,E and data not shown). At E10.5, the majority of cells within the left ventricle were β-gal positive; however, some groups of cells

FIGURE 15

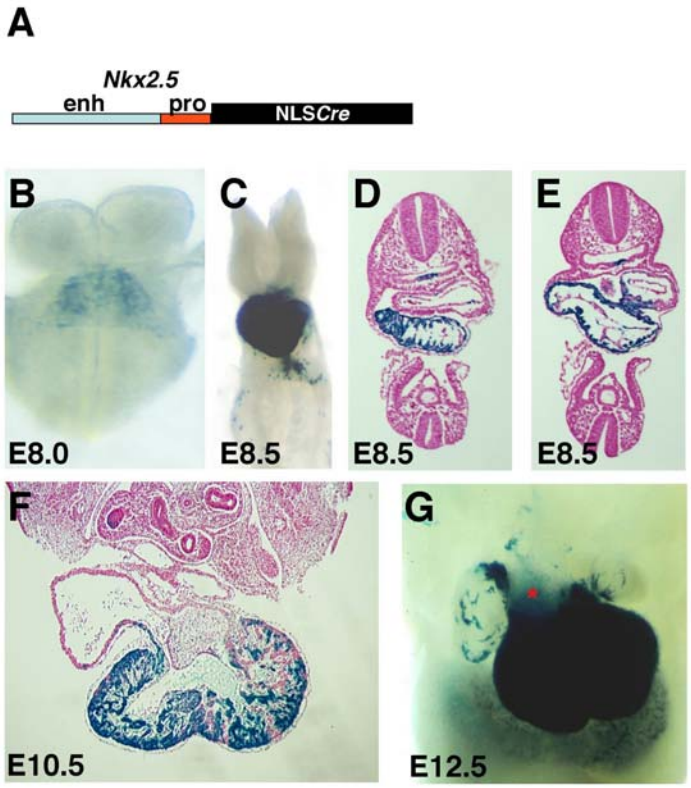


Figure 15. Generation of *Nkx2.5::Cre* mice. (A) Diagram of *Nkx2.5::Cre* transgene construct. (B-C) Whole mount photographs of X-gal stained embryos. (B) E8.0 embryo showing recombination in the linear heart tube. (C) E8.5 embryo demonstrating efficient recombination in the ventricular myocardium. (D-E) Nuclear fast red counterstained serial sections through embryo in (C). Note high levels of β-gal staining throughoutventricular myocardium. Also note staining in a subset of endocardial cells. (F) Transverse, Nuclear fast red couterstained section through E10.5 heart, note staining in all cells of right ventricle, and absence of staining in some cells within the left ventricle. (G) Whole mount X-gal stained E12.5 embryo showing efficient ventricular recombination, and minimal recombination in the outflow tract and atria.

failed to express *lacZ*, presumably due to a lack of *Cre* expression. This may reflect downregulation of the *Nkx2.5* cardiac enhancer in the left ventricle at later stages of cardiac development (Lien et al., 1999). At E12.5, whole mount X-gal staining revealed high efficiency of Cre-mediated recombination in the left and right ventricle. Interestingly, the outflow tract failed to undergo recombination (Fig. 15G, asterisk), which may reflect contribution of a secondary heart field not derived from the cardiac crescent to the outflow tract myocardium (Kelly et al., 2001; Waldo et al., 2001).

Gene dosage-sensitive redundancy of *eHAND* and *dHAND*

If the postnatal cardiac phenotype observed in *αMHC::Cre; eHAND*$^{loxP/lacZ}$ embryos resulted from delayed excision of the floxed *eHAND* allele, then, due to earlier *Cre* expression, *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ embryos would be expected to exhibit a more severe phenotype. We therefore generated and analyzed *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ mice. Interestingly, these mice were viable until 2-4 days after birth at which point they became cyanotic and died. Histologic sectioning of mutants revealed similar congenital heart defects to those observed in *αMHC::Cre; eHAND*$^{loxP/lacZ}$ embryos, including overriding aorta, ventricular septal defects, and thickened atrioventricular valves (data not shown). This result suggested that removal of *eHAND* transcripts before E8.5 did not cause embryonic lethality or exacerbate the phenotype noted in *αMHC::Cre; eHAND*$^{loxP/lacZ}$ embryos. This contrasts with tetraploid aggregation rescue of the plactental defects in *eHAND* null embryos. In these experiments,

rescued embryos died at approximately E10.5, and displayed defects in cardiac looping and morphogenesis (Riley et al., 1998).

Although it remains formally possible that eHAND function is required prior to excision of *eHAND*^{loxP} alleles, it is also possible that *dHAND* and *eHAND* act in a genetically redundant fashion during cardiac development. In contrast to *eHAND*, expression of *dHAND* is detected throughout the atrial and ventricular myocardium, although higher levels have been reported in the right ventricle (Thomas et al., 1998b). Because of the overlapping expression in the left ventricle, it is possible that *dHAND* expression may compensate for loss of *eHAND* in the left ventricular chamber. Therefore, we crossed *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ embryos into the *dHAND* heterozygous background and harvested embryos from timed matings. Interestingly, *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$*; dHAND* $^{+/-}$ embryos were not observed in litters harvested after E10.5. At or before E10.5, however, we observed the expected Mendelian ratios of all genotypes. At E10.5, *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ *;dHAND*$^{+/-}$ embryos appeared delayed and edemic relative to wild-type and *eHAND* mutant littermates. In addition, these embryos exhibited a dilated heart tube and severe pericardial edema (Fig. 16C, compare to A,B). Histologic sectioning of these embryos revealed a thin, dilated myocardium. In addition, expression of *lacZ* from the *eHAND*^{lacZ} allele was undetectable in the left ventricle of *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ *;dHAND*$^{+/-}$ embryos. These results suggested that expansion of the ventricular chambers may be perturbed *in Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ *;dHAND*$^{+/-}$ embryos.

Defects in growth of cardiac chamber myocardium from the more primitive primary myocardium of the linear hearth tube are believed to result in embryonic lethality around

FIGURE 16

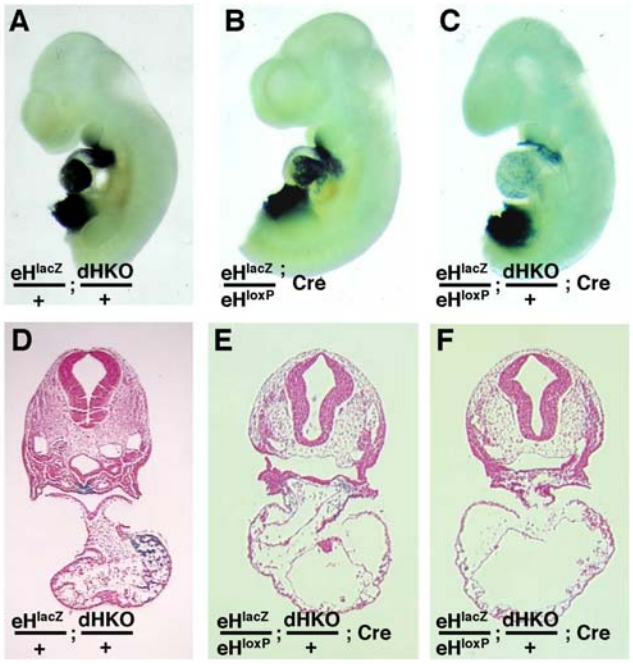


Figure 16. Abnormal cardiac morphogenesis in *Nkx2.5::Cre; eHAND$^{loxP/lacZ}$;dHAND$^{+/-}$* embryos. (A-C) Whole mount E10.5 embryos stained for β-gal activity expressed from the *eHAND^{lacZ}* allele. (A) *dHAND$^{+/-}$; eHAND$^{lacZ/loxP}$* double heterozygous embryo. (B). *Nkx2.5::Cre; eHAND$^{lacZ/loxP}$* embryo. (C) *Nkx2.5::Cre; eHAND$^{lacZ/loxP}$; dHAND$^{+/-}$* embryo showing severely reduced numbers of *eHAND* expressing cells in the heart and hypoplastic, dilated ventricular chamber. (D) Histology of *eHAND$^{lacZ/loxP}$* embryo. Note trabeculation and formation of a primitive septal outgrowth from the ventricular myocardium (arrowhead). (E,F) Histology of *Nkx2.5::Cre; eHAND$^{lacZ/loxP}$; dHAND$^{+/-}$* embryo embryo showing thin, dilated myocardium and absence of ventricular septation.

E10.5 (Christoffels et al., 2000; Yamagishi et al., 2001). Therefore, we examined expression of several markers of chamber myocardium in *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ *;dHAND*$^{+/-}$ embryos. Interestingly, expression of *ANF*, which was misregulated in *eHAND* mutant embryos, was completely absent in *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ *;dHAND*$^{+/-}$ embryos (Fig. 17A,B). In addition, *cx40* expression, that was expressed at near normal levels in *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ embryos, was completely absent from the ventricular myocardium in *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ *;dHAND*$^{+/-}$ embryos (Fig. 17 C,D). Intrigiungly, mice lacking the T-box transcription factor, *Tbx5*, also fail to express *ANF* and *cx40* (Bruneau et al., 2001). Therefore, to determine if *dHAND* and *eHAND* act genetically upstream of *Tbx5*, we examined expression of *Tbx5* in *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ *;dHAND*$^{+/-}$ embryos. Although *Tbx5* expression was reduced in the myocardium, transcripts were easily detected in the left ventricle and atrium by whole mount in situ hybridization (Fig. 17E,F). This suggests that eHAND and dHAND regulate *cx40* and *ANF* expression through mechanisms independent of *Tbx5* transcription.

FIGURE 17

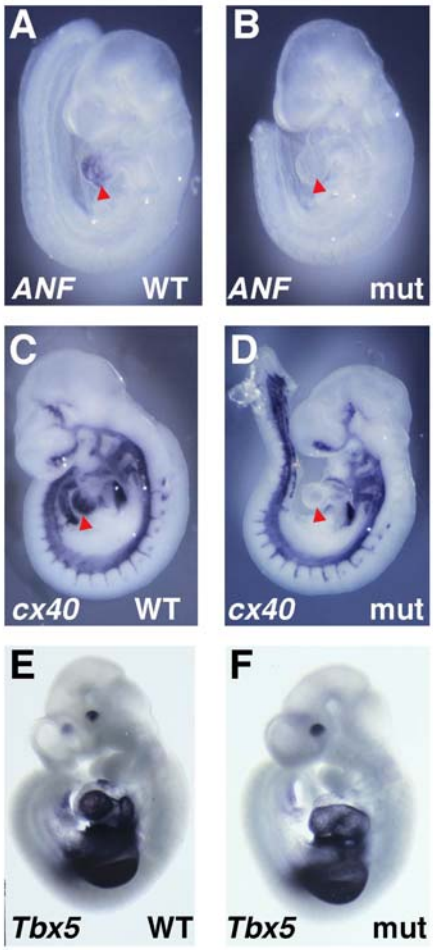


Figure 17. Downregulation of *connexin40* and *ANF* expression in *Nkx2.5::Cre; eHAND$^{loxP/lacZ}$;dHAND$^{+/-}$* embryos. Whole mount in situ hybridization of E9.5 embryos. (A-B) *ANF* expression in wild-type (A) and *Nkx2.5::Cre; eHAND$^{loxP/lacZ}$;dHAND$^{+/-}$* mutants (B). Note absence of *ANF* expression in left ventricle in mutant embryo (red arrowhead). (C-D) *connexin40* (*cx40*) expression in wild-type (C) and mutant (D) embryos. Note absence of *cx40* expression in mutant left ventricle (red arrowhead), but similar levels in dorsal aorta and vasculature. (E-F) *Tbx5* is expressed at similar levels in wild-type (E) and mutant (F) hearts. Also note similar expression pattern (LV and left atrium) in wild-type and mutant.

Discussion

The data presented here describe defects in cardiac morphogenesis observed in mice lacking myocardial *eHAND* expression. *eHAND* mutant mice die shortly after birth and display a spectrum of congenital heart defects which we presume are responsible for neonatal lethality. Interestingly, *eHAND* mutant hearts display thickened atrioventricular valves and hyperplastic endocardial cushions, implicating eHAND as an important regulator of cardiac valve formation. In addition, intercrosses of *eHAND* conditional knockout mice with *dHAND* heterozygous animals reveal gene dosage sensitive genetic redundancy of mammalian *HAND* genes.

Congenital heart defects in *αMHC::Cre; eHAND*$^{loxP/lacZ}$ embryos

The spectrum of congenital heart defects observed in *eHAND* mutant hearts suggests that eHAND is an important regulator of cardiac morphogenesis. The most frequent defect present in *eHAND* mutants is a membranous ventricular septal defect (VSD) accompanied by an overriding aorta. VSDs are the most common congenital heart defect observed in humans (Hoffman, 1995), and have been observed in a variety of targeted mouse mutants (Srivastava, 2001). Although VSDs can result from abnormal AV cushion remodeling (see below), we believe that abnormal morphogenesis of the muscular septum contributes to this phenotype in *eHAND* mutants. The presence of a widened, disorganized muscular septum at all stages of embryonic development supports this idea and suggests that growth of the septal myocytes is dysregulated. In support of this notion, we occasionally observe defects in the muscular

ventricular septum in *eHAND* mutants (data not shown). Interestingly, *eHAND* is expressed in only a subpopulation of septal myocytes; therefore, ventricular septal malformations may also be secondary to abnormal morphogenesis of the left ventricular myocardium.

Mice lacking *Nkx2.5* die at midgestation from cardiac insufficiency (Lyons et al., 1995; Tanaka et al., 1999a). The left ventricular chamber fails to expand following cardiac looping, and expression of several markers of cardiac differentiation is reduced throughout the remaining myocardium (Tanaka et al., 1999a; Yamagishi et al., 2001). Interestingly, *eHAND* expression is abolished in the hearts of *Nkx2.5*$^{-/-}$ embryos (Biben and Harvey, 1997). Because the left ventricle is more severely affected in *Nkx2.5* mutants, it has been proposed that loss of eHAND may be responsible for abnormal cardiac morphogenesis of *Nkx2.5* mutant hearts. In contrast, the data presented here suggest that lack of eHAND is not solely responsible for left ventricular hypoplasia in *Nkx2.5* null mice. First, although loss of *eHAND* expression in the early heart tube results in a diminished left ventricular chamber in the embryo, it does not result in embryonic lethality. Second, in contrast to *Nkx2.5* null mice, *ANF* and *mlc2V* are expressed at high levels in the left ventricle of eHAND mutants. Both *ANF* and *mlc2v* are also normally expressed in *eHAND*$^{lacZ/lacZ}$ null mice (Firulli et al., 1998), suggesting that expression of these markers in *eHAND* conditional knockout mice is not due to inefficient or delayed excision of *eHAND*. In addition, markers of the left ventricular chamber expansion, including *Tbx5* and *cx40*, are expressed normally in the absence of eHAND. Although a direct test of this hypothesis will require a targeted knockin mutation of *eHAND* into the *Nkx2.5* locus, we believe that lack of *eHAND* expression is only partially responsible for cardiac defects and embryonic lethality observed in *Nkx2.5* null mice.

Examination of the expression patterns of several genes during cardiac development has suggested that ventricular chamber myocardium arises from the outer curvatures of the early heart tube (Christoffels et al., 2000). Because *eHAND* is expressed along the outer curvature of the left ventricle and outflow tract, it is possible that it plays an important role in the expansion of chamber myocardium. Although left ventricular chamber size is diminished in *eHAND* mutant embryos, the left ventricle is not severely hypoplastic at birth. In addition, normal expression of *cx40, Tbx5*, and *serca2A* in *eHAND* mutants suggest that ventricular expansion is not uniformly perturbed in the absence of eHAND. Therefore, it is more likely that more subtle defects in left ventricular morphogenesis and gene expression are present in *eHAND* mutant embryos.

The transcriptional coactivator cited1 and eHAND are expressed in nearly identical patterns in the developing heart (Biben and Harvey, 1997; Dunwoodie et al., 1998). In addition, expression of *cited1* is dramatically reduced in *eHAND* mutant embryos, suggesting that *eHAND* acts upstream of *cited1*. Interestingly, mice lacking the closely related factor *cited2* display numerous congenital heart defects including ventricular and atrial septal defects, overriding aorta, double outlet right ventricle, and persistent truncus arteriosus (Bamforth et al., 2001). However, we detected no difference in *cited2* expression between wild-type and eHAND mutant mice (data not shown). Although the phenotype of *cited1* null mice has not been reported, it remains interesting to speculate that lack of cited1-dependent transcriptional coactivation may be contribute to the defects observed in *eHAND* mutants.

Novel roles of eHAND during endocardial cushion morphogenesis

An unexpected observation from our histologic analysis of *αMHC::Cre; eHAND*$^{loxP/lacZ}$ embryos was abnormally thickened atrioventricular valves and hyperplastic endocardial cushions. The endocardial cushion malformations in *αMHC::Cre; eHAND*$^{loxP/lacZ}$ mice are particularly interesting because the *αMHC::Cre* transgene does not direct *Cre* expression in the endocardial cushions or cardiac valves (Agah et al., 1997; Gaussin et al., 2002). This suggests that eHAND regulates a myocardium-derived signal that controls endocardial cushion morphogenesis. Cross-talk between the myocardium and endocardium is well documented and has been shown to involve BMP- and TGFβ-mediated signals (Brown et al., 1999; Eisenberg and Markwald, 1995; Gaussin et al., 2002; Kim et al., 2001). However, *BMP2*, *BMP4*, and *TGFβ* expression is not altered in *eHAND* mutants, suggesting that other pathways are regulated by eHAND. Because we were unable to demonstrate significant changes in gene expression and cell death in the endocardial cushions, and preliminary results indicate no changes in cell proliferation at later stages of endocardial cushion development (data not shown), it is possible that eHAND is required at an early stage of endocardial cushion morphogenesis. In support of this idea, *eHAND* mutant hearts display hyperplastic cushions before E11.5.

One interesting possibility is that eHAND may negatively regulate the epithelial to mesenchymal transformation that initiates formation of the endocardial cushions. This can be modeled in vitro using AV explants onto a collagen gel matrix (Brown et al., 1999; Dor et al., 2001; Eisenberg and Markwald, 1995). Endocardial cells that transform into mesenchyme migrate away from the explant and can be quantitated. These assays are in

progress in order to determine if eHAND regulates epithelial-mesenchymal transformation of the endocardium.

Although we believe that abnormalities of morphogenesis of the muscular ventricular septum contribute to the VSDs observed in *eHAND* mutants, the uniform presence of defects in the membranous portion of the ventricular septum suggests that defective AV cushion morphogenesis is also involved. Portions of the AV cushions contribute to both the atrial and ventricular septa, and alterations in AV cushion remodeling result in predictable septal defects involving the membranous ventricular septum (Bartram et al., 2001). Therefore, defects in both AV cushion and septal myocardium are likely to contribute to the VSDs observed in *eHAND* mutants.

Redundancy of *HAND* genes during cardiac morphogenesis

Generation of *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ *;dHAND*$^{+/-}$ embryos has enabled us to demonstrate genetic redundancy between mammalian *dHAND* and *eHAND*. These embryos arrest in development and die around E10.5 from apparent cardiac insufficiency. In contrast to embryos lacking dHAND, which die around E10.5 and exhibit a severely hypoplastic right ventricle (Srivastava et al., 1997), *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ *;dHAND*$^{+/-}$ embryos appear to develop myocardium from both the left and right ventricular chambers, as evidenced by the normal cardiac form and *Tbx5* expression in these embryos. In addition, normal expression of *mlc2V* and *serca2A* suggests that myocardial differentiation and chamber expansion are not completely abrogated in these animals. However, the ventricular myocardium appears thin relative to wild-type embryos. The cellular mechanism responsible for these defects is

currently unclear. We are currently examining cell death and proliferation, as well as expression of candidate downstream target genes in these animals. The exquisite sensitivity of *eHAND* mutant hearts to *dHAND* gene dosage was unanticipated, and underscores the critical role of these genes during cardiac morphogenesis. Gene expression analyses in these animals should facilitate identification HAND target genes expressed in the myocardium would not otherwise be easily identified in *dHAND* or *eHAND* null mice. In addition, it will also be important to determine if *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ *;dHAND*$^{-/-}$ mice also exhibit a more severe phenotype than *dHAND*$^{-/-}$ embryos.

Expression of *ANF* and *cx40* was completely abolished in *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$*; dHAND*$^{+/-}$ embryos. Interestingly, *ANF* and *cx40* expression is also absent in mice lacking Tbx5 (Bruneau et al., 2001). In addition, mice heterozygous for *Tbx5* upregulate *ANF* in the right ventricle, similar to the expression we noted in *eHAND* mutant hearts (Fig 12G,H). Therefore it is possible that eHAND regulates *ANF* and *cx40* expression via transcriptional activation of *Tbx5*. However, *Tbx5* is expressed at normal levels in *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$ *;dHAND*$^{+/-}$ embryos. As both E-boxes and T-box binding sites are present in the *cx40* and *ANF* promoters, it is interesting to speculate that eHAND and Tbx5 synergistically regulate expression of *cx40* and *ANF*. It is also of note that Tbx5 and Nkx2.5 have been shown to physically interact and synergistically activate the *cx40* and *ANF* promoters (Bruneau et al., 2001; Hiroi et al., 2001). Experiments to examine potential transcriptional activation by eHAND on these transcriptional regulatory elements are currently underway.

Generation of mice lacking myocardial expression of *eHAND* has identified a novel function for eHAND in regulation of endocardial cushion morphogenesis and ventricular septation. In addition, intercrosses of *eHAND* conditional knockout with *dHAND* heterozygous mice have demonstrated genetic redundancy between mammalian *HAND* genes during heart development. It will be important to identify the eHAND-dependent signals from the myocardium that control morphogenesis of the endocardial cushions, and to identify target genes expressed in the myocardium that mediate the combined actions of eHAND and dHAND during heart morphogenesis.

Methods

Gene Targeting

A previously characterized *eHAND* genomic clone (Firulli et al., 1998), was used to generate the *eHAND*^{loxP} targeting vector. A 2.9 kb fragment extending upstream from the *eHAND* 5'-UTR (long arm of homology) was amplified by high-fidelity PCR (Stratagene *Pfu* Turbo) and digested with *Xho*I. An *Eco*RI restriction site was engineered into the *eHAND* 5'UTR to facilitate genotyping by Southern blotting. This fragment was ligated into the pDelboy targeting backbone upstream of the FRT-flanked neomycin resistance cassette. (gift of M. Henkemeyer). A *Cla*I-*Kpn*I linked fragment containing the first exon of *eHAND*, extending from the *eHAND* 5'-UTR to the intron, was amplified using high fidelity PCR and cloned into pDelboy-LA. Finally, the short arm of homology, extending from the intron to a 3' *Sal*I site, was PCR amplified and cloned into *Sal*I-*Eco*RI digested pDelboy-LAeH upstream of the thymidine kinase negative selection cassette. Integrity of the targeting vector was confirmed by restriction mapping and DNA sequencing. PCR primer sequences are available upon request.

The completed *eHAND*$^{NEO\text{-}loxP}$ targeting vector was linearized with *Not*I and electroporated into SM-1 ES cells (gift of Masashi Yanagisawa and Robert Hammer). Following positive-negative selection with G418 and FIAU, resistant colonies were screened by Southern analysis of *Eco*RI digested genomic DNA using a probe (Figure 8) from the 3' flanking region. Recombination of the 5' arm was confirmed by *Eco*RI-*Kpn*I double digestion of genomic DNA, and Southern blotting with the short arm of homology. Three

correctly targeted clones (clones E12, C12 and C5) were expanded and injected into C57BL/6 blastocysts, and transferred into the uteri of pseudopregnant females. Chimeric males were bred onto a C57BL/6 or Black/Swiss background for germline transmission. Males from clone E12 tramsmitted the targeted allele through the germline, therefore mice derived from this line were used in all analyses.

Heterozygous *eHAND*$^{NEO\text{-}loxP}$ mice were intercrossed with *hACTB::FLPe* transgenic mice (Rodriguez et al., 2000)(gift of Susan Dymecki) in order to remove the neomycin resistance cassette in the germline. Removal of the neomycin cassette was confirmed by Southern blotting using *Eco*RI digested genomic DNA (Figure 8). The *eHAND*^{loxP} allele was bred to homozygosity, which did not affect viability or fertility of these mice, suggesting that the FRT and loxP sites in the 5'-UTR did not significantly alter expression from the targeted locus.

Generation of *Nkx2.5::Cre* mice

A 2.5kb fragment containing the *Nkx2.5* basal promoter and cardiac enhancer (Lien et al., 1999) was cloned upstream of the NLS-Cre expression cassette (gift of J. Herz). This vector was linearized using *Not*I and injected into fertilized oocytes as previously described (McFadden et al., 2000). Founder transgenic mice were genotyped by hybridization of *Eco*RV digested tail DNA to a *Cre* cDNA probe. Three transgenic lines were obtained and intercrossed with *ROSA26R* indicator mice (Soriano, 1999) in order to assess transgene expression and Cre-mediated recombination. Transgenic line Nk9 exhibited the earliest and most efficient recombination, and was used in all subsequent experiments.

PCR Genotyping

Tail and yolk sac DNA was isolated as previously described (McFadden et al., 2000). PCR reactions were used to genotype Cre transgenes, and *eHAND* and *dHAND* knockout loci (Firulli et al., 1998; Srivastava et al., 1997). Briefly, 1μL of tail or yolk sac DNA was used a template in 25μL PCR reactions using Promega *Taq* polymerase and 4mM $MgCl_2$. Thermal cycle reactions were as follows: 2 min 95°C, 30 cycles of 30s 95°C, 30s 55°C, 45s 72°C, and a final 5 minute extension at 72°C. Reactions were visualized on 1% agarose gels in TAE. Primer sequences are available upon request.

RT-PCR

Left ventricles from E9.5 embryos were dissected and immediately frozen and stored in liquid nitrogen until embryo and yolk sac DNA was isolated and genotyped. Left ventricular tissue from 8 mutant hearts was pooled and total RNA was isolated using Trizol reagent and standard protocols. 150 ng of total LV RNA was used as a template for first strand cDNA synthesis using the Superscript first strand synthesis kit from Invitrogen. 1/20 of the cDNA synthesis reaction was used as template for PCR reactions using Promega *Taq* polymerase to detect *eHAND* transcripts. Thermal cycles were as follows: 94°C 2min, 28 cycles of 94°C 30s, 52°C 30s, 72°C, 30s, and a final 5 minute extension at 72°C. Reactions were visualized on 1% agarose gels in TAE. Primer sequences are available upon request.

Histology

Embryos were harvested from timed matings and fixed overnight in 4% paraformaldehyde in 1X phosphate buffered saline (PBS). Following fixation, embryos were rinsed in 1X PBS then dehydrated through graded ethanols and embedded in paraffin as previously described (Moller and Moller, 1994). Histologic sections were cut and stained with hematoxylin and eosin, or nuclear fast red as previously described (Moller and Moller, 1994).

In situ hybridization

Section in situ hybridization was performed as described (Shelton et al., 2000). Whole mount in situ hybridization was performed as previously described (Riddle et al., 1993). Plasmids for in situ probes have been previously described and were linearized and transcribed as follows: *ANF*: *Xho*I, T7, *BMP2*: *Xba*I, T3, *BMP4*: *Eco*RI, SP6, *Smad6*, *Smad7*, *TGF*β_2: *Eco*RI, SP6, *connexin 40*: *Asp*718, T3, *connexin 43*: *Sal*I, T7, *Serca2A*: *Xho*I, T3, *Tbx5*: *Spe*I, T7, *mlc2V*: *Bam*HI, T7, *Tbx2*. The coding regions of *cited1* and *cited2* were amplified as *Cla*I-*Eco*RI fragments and subcloned into pBSK. Both plasmids were linearized with *Xho*I and transcribed with T3 RNA polymerase. *dHAND* and *eHAND* coding regions were amplified as *Eco*RI-*Xba*I fragments and cloned into pBSK. Both plasmids were linearized with *Eco*RI and transcribed with T7.

β-galactosidase staining

Embryos from timed matings were harvested and pre-fixed for 1-3 hours in 2% paraformaldehyde, 0.25% glutaraldehyde in 1X PBS. Staining for β-gal activity was performed as previously described (McFadden et al., 2000).

TUNEL and Immunohistochemistry

TUNEL staining was performed on paraffin embedded sections from E10.5 and E13.5 according to the Promega Fluorescein Apoptosis detection kit.

Embryos were harvested at E11.5 and fixed overnight in 4% paraformaldehyde in 1X PBS. Embryos were rinsed into 1X PBS, and equilibrated into 10% sucrose for 2 hours, followed by 30% sucrose overnight at 4°C. Embryos were transferred into OCT freezing medium and frozen in isopentane and liquid nitrogen. Blocks were equilibrated to -20°C and serially sectioned. Sections were stored at -80°C until antibody staining.

Antibody staining was performed as described (Frey et al., 2000). Primary α-phospho histone H3 antibody was diluted 1:200 in 1%BSA in 1X PBS.

CHAPTER 4: MISEXPRESSION OF dHAND CAN INDUCE ECTOPIC DIGITS IN THE ABSENCE OF DIRECT DNA BINDING

Introduction

Studies of the vertebrate limb have yielded important insights into the mechanisms for embryonic patterning along the dorsoventral, anteroposterior (A-P), and proximodistal axes (Johnson and Tabin, 1997). Growth and patterning of the limb bud along the A-P axis is controlled by the zone of polarizing activity (ZPA), a specialized region of mesoderm at the posterior margin of the limb bud. Grafts of the ZPA into the anterior margin of a recipient limb bud result in mirror-image duplications of the distal skeletal elements (Saunders, 1968). The secreted protein Sonic hedgehog (SHH) is localized to the ZPA, and application of beads soaked in recombinant SHH to the anterior margin of the limb results in mirror-image digit duplications (Riddle et al., 1993). Limbs from *Shh* mutant mice also show severe defects in patterning along the A-P and dorsoventral axes and lack polarizing activity in grafting experiments (Chiang et al., 2001; Chiang et al., 1996).

While SHH is a key mediator of ZPA activity, several lines of evidence indicate that it is not the sole determinant of A-P information within the limb field. The chick *limbless* mutant, for example, does not detectably express SHH, but displays nested A-P expression of 5' *Hoxd* genes within the limb (Grieshammer et al., 1996; Noramly et al., 1996; Ros et al., 1996). Limb buds from *Shh* mutant mice also retain graded A-P expression of some components of the SHH/FGF feedback loop, and form autopodial structures with recognizable A-P polarity (Chiang et al., 2001; Zuniga et al., 1999). In light of these

findings, there has been intense interest in identifying factors that act upstream of SHH to establish the ZPA.

The basic helix-loop-helix (bHLH) transcription factor dHAND/HAND2 is expressed in the posterior limb mesenchyme prior to SHH, and its expression pattern completely encompasses that of SHH at all later stages of limb development (Charité et al., 2000). Moreover, limb buds from mice and zebrafish lacking dHAND fail to upregulate SHH expression (Charité et al., 2000; Yelon et al., 2000). Misexpression of dHAND in the anterior limb mesoderm of mouse or chick embryos also is sufficient to induce ectopic SHH and mirror image duplications of posterior skeletal elements (Charité et al., 2000; Fernandez-Teran et al., 2000).

A recent study has further defined the role of dHAND in prepatterning the A-P axis of the limb bud (te Welscher et al., 2002). The transcriptional repressor GLI3 is expressed in the anterior limb mesenchyme, and plays an important role in positioning the ZPA at the posterior margin of the limb bud (Zuniga and Zeller, 1999). In the polydactylous mouse mutant *Extratoes* (*Xt*), which disrupts *Gli3* (Buscher et al., 1997; Hui and Joyner, 1993; Schimmang et al., 1992), *dHAND* expression persists in the anterior mesenchyme, demonstrating that GLI3 represses transcription of *dHAND* during limb bud outgrowth. Conversely, in *dHAND* $^{-/-}$ embryos, expression of *Gli3* and the *Aristaless-like4* (*Alx4*) gene, which encodes a transcriptional repressor, are expanded into the posterior limb mesenchyme. Misexpression of dHAND in the anterior mesoderm is also sufficient to induce the BMP antagonist Gremlin, an important component of the SHH/FGF feedback loop that maintains the ZPA (Zuniga et al., 1999). Therefore, it appears that dHAND acts both positively to

induce ZPA formation, as well as negatively to repress expression of factors that inhibit ZPA formation. The fact that dHAND is required for the activation of *Shh*, *Hoxd11*, *Hoxd12* and *BMP2*, and repression of *Gli3* and *Alx4* which are key regulators of A-P patterning (Charité et al., 2000; Fernandez-Teran et al., 2000; te Welscher et al., 2002; Yelon et al., 2000), suggests that it may act as both a transcriptional activator and repressor. It remains unclear, however, which, if any, of these genes represent direct transcriptional targets of dHAND.

Members of the bHLH family of transcription factors regulate the specification and differentiation of numerous cell types during embryonic development (Massari and Murre, 2000). In general, tissue-specific (class B) bHLH factors form obligate heterodimers with ubiquitous bHLH proteins (class A), called E-proteins. Dimerization juxtaposes the basic regions of bHLH proteins to form a bipartite DNA binding domain that recognizes the E-box consensus sequence (CANNTG) with resulting transcriptional activation or repression of target genes.

dHAND and the closely related bHLH protein eHAND are coexpressed in many embryonic cell types, although only dHAND is expressed in the developing limb bud (Charité et al., 2000; Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995; Srivastava et al., 1995). eHAND has been shown to bind a nonconsensus E-box sequence (NNTCTG) as a heterodimer with the E-protein E12. Reporter gene assays in tissue culture cells have suggested that eHAND may have dual functions as both a transcriptional repressor and activator (Hollenberg et al., 1995; Scott et al., 2000). However, the potential transcriptional activities of dHAND have not been reported.

To further understand the mechanisms whereby dHAND regulates transcription during limb development, we performed a structure-function analysis of the dHAND protein in cultured cells and misexpressed dHAND mutant proteins in the developing limb buds of transgenic mice. Remarkably, our results show that only the HLH motif of dHAND is required to induce the formation of ectopic digits. This activity is thus independent of direct DNA binding and the basic region, as well as the amino-terminal transactivation domain. Digit duplications are also induced by eHAND, but not by the related bHLH protein paraxis, indicating that the highly conserved HLH regions of dHAND and eHAND have unique functions not shared by other bHLH family members. These findings reveal an unanticipated specificity of the HLH region of dHAND as a regulator of tissue morphogenesis and patterning and suggest that dHAND may govern these processes through a mechanism independent of direct DNA binding.

Results

dHAND activates transcription through a consensus E-box.

To begin to investigate the transcriptional properties of dHAND, we tested whether the *in vitro* translated protein was able to bind a consensus E-box identified in site selection experiments with eHAND (Hollenberg et al., 1995). dHAND alone did not show significant binding to this site in gel mobility shift assays, but in the presence of E12, a prominent DNA-protein complex was observed, reflecting the binding of dHAND-E12 heterodimers (Fig. 18A).

To determine the transcriptional consequences of dHAND binding to this site, we tested whether dHAND could activate a luciferase reporter linked to 6 copies of this E-box (Fig. 18B). dHAND alone evoked only a minimal increase in luciferase activity in transfected 10T1/2 cells. Similarly, E12 failed to substantially activate this reporter. However, cotransfection of dHAND and E12 expression vectors resulted in approximately 65-fold activation of the reporter. These results suggest that the level of expression of dHAND alone in transfected cells exceeds the level of endogenous E-proteins, therefore limiting activation of the reporter, and that providing additional E12 facilitates heterodimer formation and subsequent DNA binding and transcriptional activation.

In order to identify the regions of dHAND that contribute to its transcriptional activity, we generated a series of deletions of the dHAND coding region (Fig. 18B and Fig. 19). All dHAND proteins contained an amino-terminal Myc epitope tag that allowed their detection by western blotting. Mutant proteins were expressed at levels comparable to the

FIGURE 18

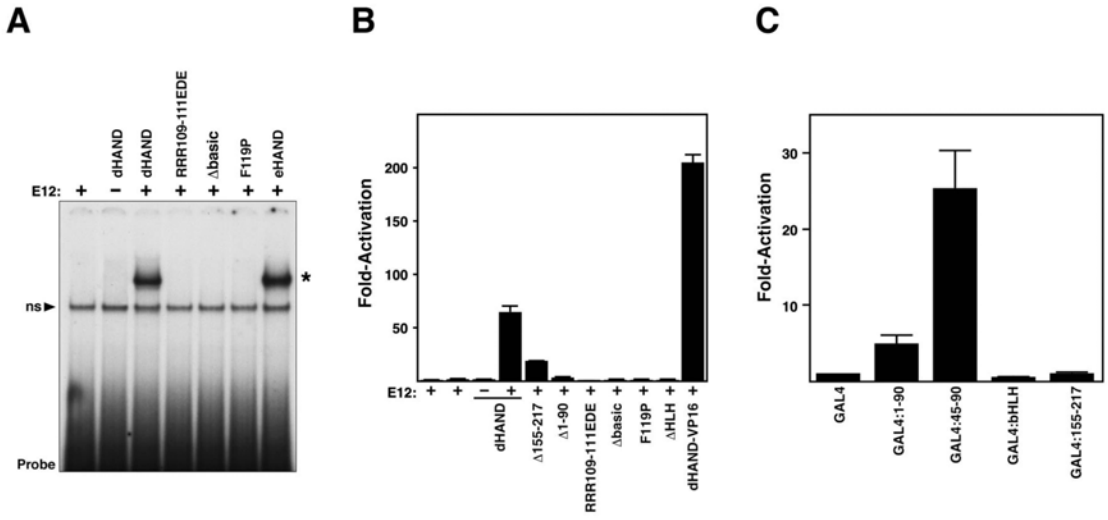


Fig. 18. DNA binding and transcriptional activity of dHAND mutant proteins. A) Wild-type and mutant dHAND proteins were translated in vitro with or without E12, as indicated, and tested for binding in gel mobility shift assays to a radiolabeled oligonucleotide probe containing the eHAND/Th-1 E-box sequence. HAND/E12 complex mobility is indicated by an asterisk and a nonspecific complex is marked by ns. B) 10T1/2 cells were transiently transfected with the L8E6-luciferase reporter and pcDNA3.1 expression vector encoding wild-type and mutant dHAND proteins (see Fig. 2) and luciferase activity was determined in cell extracts. Values are expressed as fold-activation of the reporter gene relative to the level of expression with the reporter alone. C) 10T/2 cells were transiently transfected with the L8G4-luciferase reporter and the pGE1b-GAL4 expression vector encoding portions of the dHAND coding region fused to the GAL4 DNA binding domain and luciferase activity was determined in cell extracts. Values in B and C represent the mean +/- standard error determined from at least three independent experiments.

FIGURE 19

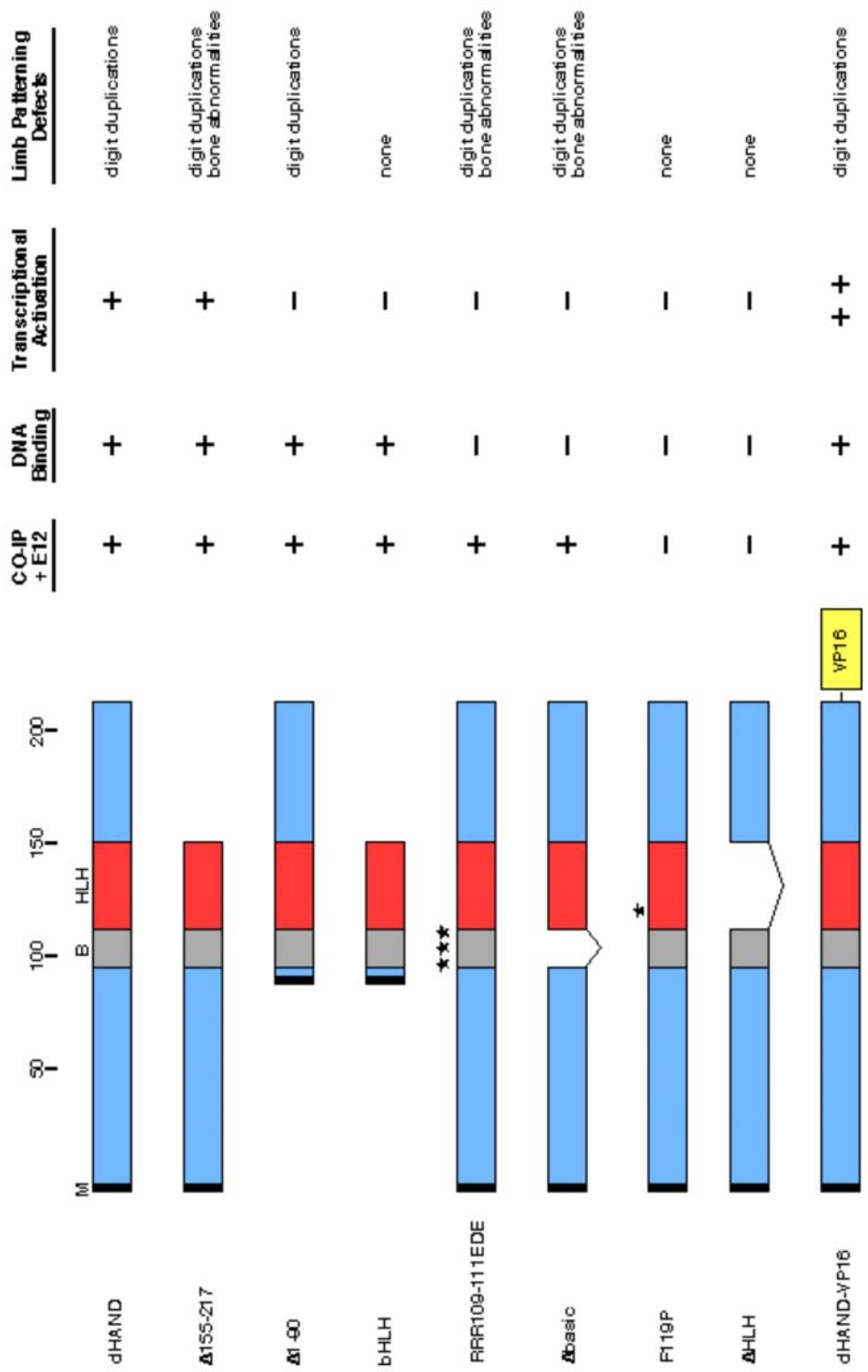

Limb Patterning Defects
Transcriptional Activation
DNA Binding
CO-IP + E12
digit duplications
digit duplications bone abnormalities
digit duplications
none
digit duplications bone abnormalities
digit duplications bone abnormalities
none
none
digit duplications
VP16
M
50
100
150
200
B
HLH
dHAND
Δ155-217
Δ1-90
bHLH
RRR109-111EDE
Δbasic
F119P
ΔHLH
dHAND-VP16

Figure 19. Summary of the activities of dHAND mutant proteins. Mutant dHAND proteins and their activities are shown. All mutants contained an amino-terminal myc epitope tag. M, myc epitope tag; B, basic region; HLH, helix-loop-helix.

wild-type protein (data not shown). To confirm that mutant proteins folded properly and were functional *in vivo,* each was also tested by co-immunoprecipitation with E12, and for DNA binding in gel mobility shift assays (Fig. 18A, Fig. 19, and data not shown).

Because the carboxy terminal amino acids of dHAND are highly conserved among all orthologs of HAND proteins (Angelo et al., 2000), we speculated that this region might be important for the transcriptional activity of dHAND in these reporter assays. Deletion of this region (155-217) resulted in a 3-fold reduction of the transcriptional activity of dHAND. In contrast, deletion of amino acids 1-90 resulted in a near-complete loss of transcriptional activity. These findings suggested that the amino-terminal region of dHAND acts as a transcriptional activation domain and is responsible for the majority of dHAND's transcriptional activity, although the carboxy terminal residues appear to contribute to the transactivation potential of dHAND.

To confirm that amino acids 1-90 of dHAND can act as a transcriptional activation domain, we generated fusions of dHAND to the DNA-binding domain of the yeast GAL4 protein and tested these constructs for their abilities to activate a GAL4-dependent luciferase reporter (Fig. 18C). Residues 1-90 were able to stimulate transcription, whereas the bHLH region or carboxy terminus (residues 155-217) was not. Further deletion of the first 90 residues revealed that the most potent activation function mapped to amino acids 45-90.

Transactivation-defective dHAND mutants induce ectopic digits in transgenic mice

Recently, we and others have shown that misexpression of dHAND in the anterior portion of the limb buds results in ectopic expression of SHH at the anterior margin of the limb bud

with resulting preaxial polydactyly at later stages of development (Charité et al., 2000; Fernandez-Teran et al., 2000). To determine if there was a correlation between the limb patterning activity of dHAND and the transcriptional activity detected in tissue culture, we expressed each of the mutant proteins described above in transgenic mouse embryos using regulatory sequences from the *prx1* gene, which direct expression throughout the forelimb bud and in the anterior mesoderm of the hindlimb at E10.5 (Martin and Olson, 2000). We used the presence of ectopic digits at embryonic day 16.5 (E16.5) as a functional readout of the activities of these mutant proteins.

Because our previous studies of dHAND misexpression in the limb used a *dHAND* genomic fragment containing the single intron within the coding region (Charité et al., 2000), we first tested whether expression of a cDNA encoding a dHAND protein with an amino-terminal Myc-tag was capable of inducing ectopic digits. This dHAND cDNA effectively induced preaxial polydactyly of fore- and hindlimbs (Fig. 20A,B and data not shown). Polydactyly ranged from a single ectopic triphalangeal digit, to three ectopic digits replacing digit I. The severity and frequency of this limb phenotype were comparable to previous results using the *dHAND* genomic fragment linked to the *prx1* promoter.

Surprisingly, preaxial polydactyly was also observed in 9/12 embryos with a deletion mutant lacking the amino-terminal transcription activation domain (mutant 1-90, Fig. 20C). However, 3/12 embryos harboring this transgene showed small hypoplastic limbs with a reduced number of digits (data not shown), a phenotype we have not observed with the wild-type protein. Whether this phenotype reflects a weak dominant negative activity of the mutant protein and variability in transgene expression is unclear. Nevertheless, the presence

FIGURE 20

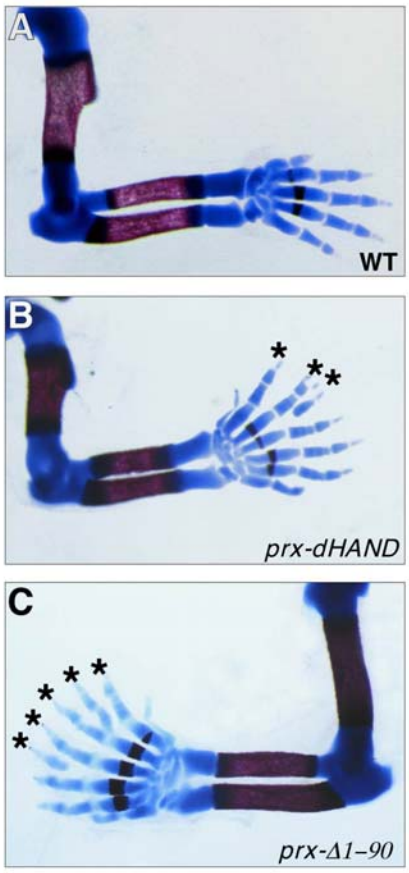


Fig. 20 Preaxial polydactyly induced by a dHAND mutant protein lacking the amino-terminal transcription activation domain. F0 transgenic mouse embryos were sacrificed at E16.5 and limbs were stained for bone and cartilage. Panel A shows a limb from a wild-type (WT) nontransgenic embryo. Embryos harboring *prx-dHAND* (B) and *prx-Δ1-90* (C) transgenes showed preaxial polydactyly. All panels show forelimbs. Ectopic digits are indicated with an asterisk.

of ectopic digits in the majority of mutant 1-90 transgenic embryos suggests that dHAND can function in the absence of the amino-terminal transcriptional activation domain.

Polydactyly in *prx-dHAND-VP16* transgenic embryos

In principle, misexpression of dHAND could repattern the limb by acting as a transactivator to induce the expression of limb patterning genes, such as *Shh*, or by repressing a repressor of posterior identity in the anterior compartment of the limb. This was an especially interesting issue in light of our results, which indicated that the amino-terminal transcriptional activation domain of dHAND was dispensable for activity of the protein in our misexpression assay. In addition, recent evidence suggests that dHAND may act as both a transcriptional activator and repressor during limb patterning. dHAND has been demonstrated to repress the transcription of *Gli3* and *Alx4* in the posterior limb mesenchyme prior to *Shh* expression (te Welscher et al., 2002). In addition, dHAND has been shown to be required for activation of *Shh*, *Hoxd11*, *Hoxd12*, and *BMP2* expression during limb and fin development (Charité et al., 2000; Fernandez-Teran et al., 2000; Yelon et al., 2000). Notably, eHAND has also been shown to act as a repressor or an activator depending on the assay (Hollenberg et al., 1995; Scott et al., 2000).

To further assess the importance of transcriptional activity of dHAND for its digit-duplicating activity, we fused the viral coactivator VP16 to the carboxy terminus, reasoning that the VP16 activation domain would interfere with this activity if dHAND acted as a transcriptional repressor. This dHAND-VP16 fusion protein was approximately 3-fold more active than the wild-type dHAND protein in reporter assays (Fig. 18B). Seven out of eight

embryos harboring the *prx-dHAND-VP16* transgene exhibited extreme preaxial polydactyly (Fig. 21A). This suggested that dHAND may act primarily through the activation of downstream target genes during limb development, although other interpretations are also consistent with this result.

Interestingly, in several *prx-dHAND-VP16* transgenic embryos, the number and morphology of the ectopic digits appeared similar to the *Doublefoot* (*Dbf*) mutant phenotype. This was also occasionally observed with other *prx-dHAND* transgenes (For examples, see Fig. 20C and Fig. 21A). In contrast to other polydactylous mutants, *Dbf* mice generally display a greater number of ectopic digits that lack distinctive A-P identity (Lyon et al., 1996). *Dbf* mutants also exhibit a broad domain of ectopic *Indian hedgehog* (*Ihh*) expression along the A-P margin of the limb (Yang et al., 1998). This differs from other polydactylous mutants, as well as transgenic embryos misexpressing of *Hoxb8* and *Hoxd12,* which display a small ectopic domain of *Shh* at the anterior margin of the limb bud (Charité et al., 1994; Knezevic et al., 1997). It is believed that the broadened expression of *Ihh,* and subsequent activation of the hedgehog signaling pathway, is responsible for the increased digit number and altered morphology (Yang et al., 1998). Although we occasionally noted *Dbf*-like phenotypes in our experiments, this was most frequently observed in *prx-dHAND-VP16* transgenic embryos.

In order to determine if a broadened ectopic *Shh* expression domain was a potential explanation for the *Dbf*-like phenotype, we examined expression of *Shh* in early stage *prx-dHAND-VP16* embryos. Whole-mount *in situ* hybridization of transgenic embryos at E11.5

FIGURE 21

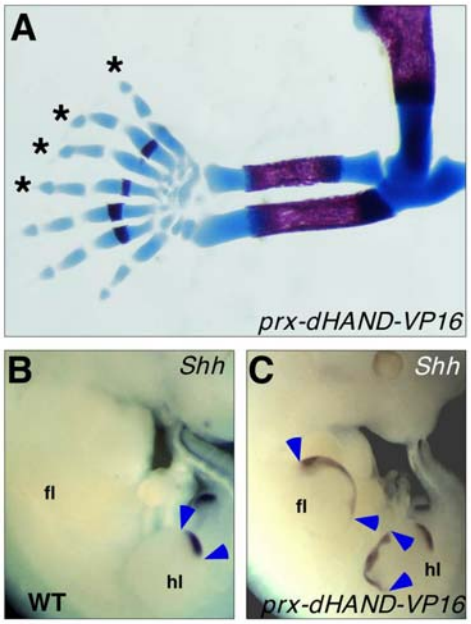


Fig. 21. Preaxial polydactyly and ectopic *Shh* expression induced by dHAND-VP16. F0 transgenic mouse embryos were sacrificed at E16.5 and limbs were stained for bone and cartilage. Panel A shows a limb from an embryo harboring the *prx-dHAND-VP16* transgene. This construct induced severe preaxial polydactyly. Note the presense of four ectopic digits which have similar morphology. B and C) Detection of *Shh* transcripts by whole-mount in situ hybridization to wild-type and *prx-dHAND-VP16* transgenic embryos, respectively, at E11.5. In wild-type embryos, *Shh* transcripts are localized to the ZPA at the posterior of the limb bud. In *prx-dHAND-VP16* embryos, *Shh* transcripts are seen throughout the peripheral region of the limb bud. Blue arrowheads indicate *Shh* expression domain. Ectopic digits are indicated with an asterisk. fl, forelimb bud; hl, hindlimb bud.

showed that this construct induced ectopic expression of *Shh* not only at the anterior margin of the limb bud, but also at intervening regions along the A-P axis of the limb bud (Fig. 21B,C). Although the expression pattern directed by the *prx1* regulatory elements becomes more complex at later stages of limb development, expression spans the A-P dimension of the limb bud through E10.5 (Martin and Olson, 2000). Therefore, the broad domain of ectopic *Shh* expression we detected one day later at E11.5 is consistent with the transgene expression at E10.5. The *Shh* expression pattern varied within individual *prx-dHAND-VP16* transgenic embryos, and between different transgenic embryos (data not shown), suggesting that integration effects as well as transgene expression levels affect both the pattern and level of ectopic *Shh* expression. Nonetheless, the presence of *Shh* transcripts throughout the A-P axis of the limbs provides a possible explanation for the *Doublefoot*-like phenotype occasionally observed in transgenic embryos.

Polydactyly and abnormal bone formation in *prx-Δ155-217* transgenic embryos

The striking evolutionary conservation at the carboxy terminus of all orthologs of HAND proteins suggested that these amino acids might be important for an activity of dHAND that was undetectable in transcriptional reporter assays. Therefore, we generated transgenic embryos expressing a mutant that lacked this region (mutant Δ155-217). These embryos also exhibited preaxial polydactyly (Fig. 22A). However, in contrast to the phenotype observed with the wild-type dHAND protein, bones of the zeugopod, in both forelimbs and hind limbs, were shortened and malformed. There was a direct correlation between the severity of the

polydactyly and zeugopod truncation, suggesting that the variability in severity of these abnormalities reflected the expression level of the transgene.

It is interesting to consider how misexpression of dHAND mutants under the control of the *prx1* promoter might result in zeugopod abnormalities, given that the *prx1* promoter is not active in the zeugopod per se. At least two observations may relate to this phenomenon. First, *prx1* is expressed throughout the early limb bud (Martin and Olson, 2000). Thus, it is possible that misexpression of dHAND mutants at this early stage results in abnormalities observed later in the zeugopod. Second, at later stages of limb development, endogenous *prx1* is expressed in the periosteum surrounding the bones of the zeugopod (Cserjesi et al., 1992). The *prx1* promoter fragment also directs expression in the periosteum and tendons at later stages of limb development (Martin and Olson, 2000). In support of these notions, bones of the zeugopod are malformed in *prx1* $^{-/-}$ mice, suggesting that *prx1* expression either throughout the early limb, or later in the periosteum, is required for normal bone morphogenesis, and that misexpression of proteins in these tissues can affect bone growth and morphogenesis (Martin et al., 1995).

The zeugopod phenotype observed with mutant Δ155-217 suggested that dHAND might play a role in bone growth or differentiation during late stages of limb development. We therefore examined the expression pattern of *dHAND* in developing limb bones of wild-type embryos from E12.5-E15.5 by *in situ* hybridization. As shown in Fig. 22B, *dHAND* was expressed at E15.5 in the chondrocytes and perichondrium. The highest levels of *dHAND* expression were noted in the hyperplastic zone of chondrocytes in the ulna. These findings raise the possibility that the phenotype of the zeugopod in *prx-Δ155-217* transgenic embryos

FIGURE 22

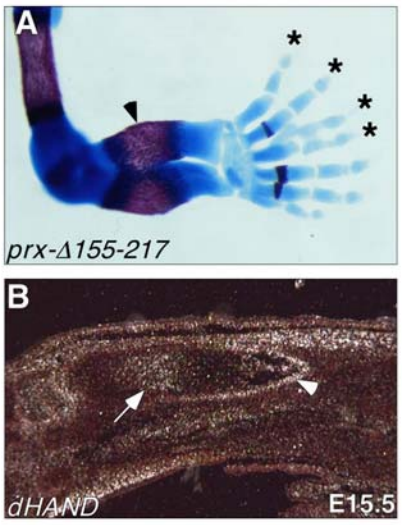


Fig. 22 . Limb abnormalities induced by a dHAND mutant protein lacking the carboxy terminal region. F0 transgenic mouse embryos were sacrificed at E16.5 and limbs were stained for bone and cartilage. Panel A shows a forelimb from an embryo harboring *prx-Δ155-217*. This construct induced preaxial polydactyly, as well as truncation of the zeugopod (indicated by black arrowhead). B) Detection of *dHAND* transcripts by in situ hybridization to a longitudinal section through the developing ulna of an E13.5 embryo. *dHAND* expression is detected in the chondrocytes and perichondrium (white arrowhead). Note highest levels of expression in the hyperplastic zone of the chondrocytes (white arrow). Ectopic digits are indicated with an asterisk.

may reflect a dominant negative action of this truncated protein at some stage of bone development. However, since this mutant retained the ability to induce polydactyly with altered A-P patterning, we focused on identifying the region of dHAND responsible for this activity.

DNA-binding independent functions of dHAND

In order to determine if direct DNA binding is required for dHAND activity in our transgenic assay, we generated mutations in the basic region that abolished DNA binding in vitro (Fig. 23A). Mutation of three conserved asparagine residues to acidic amino acids (RRR109-111EDE) completely abolished DNA binding activity of dHAND in vitro (Fig. 18A). Surprisingly, transgenic mouse embryos harboring a *prx-RRR109-111EDE* transgene exhibited preaxial polydactyly, as well as malformed bones in the zeugopod, similar to those seen in *prx-Δ155-217* transgenics (Fig. 23B, compare to Fig. 21A). To eliminate the possibility that the RRR109-111EDE mutant might bind DNA weakly or recognize undefined DNA binding sites in vivo, we deleted the entire basic region (mutant Δbasic). Embryos harboring the *prx-Δbasic* transgene also exhibited polydactyly and malformed bones of the zeugopod; this phenotype was indistinguishable from those of *prx-RRR109-111EDE* and *prx-Δ155-217* transgenic embryos (Fig. 23C). Together, these results provide evidence that dHAND may not require, at least in some developmental contexts, direct DNA binding in order to modulate downstream target genes.

FIGURE 23

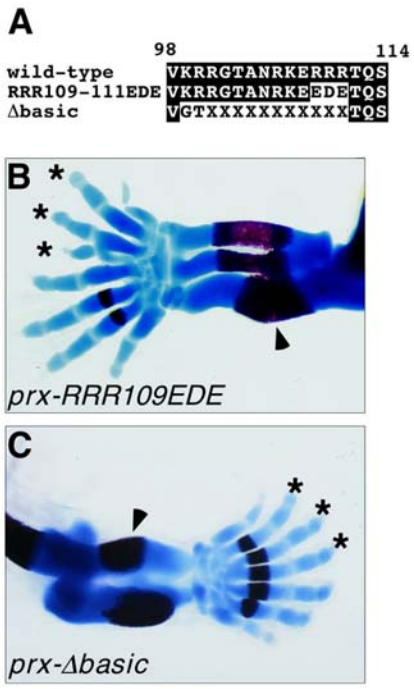


Fig. 23. Limb abnormalities induced by dHAND mutant proteins lacking the basic region.
A) Amino acid sequence of the basic regions of wild-type and mutant dHAND proteins. X designates residues that were deleted in the Δbasic mutant. B and C) F0 transgenic mouse embryos harboring *prx-RRR109EDE* and *prx-Δbasic* transgenes, respectively, were sacrificed at E16.5 and limbs were stained for bone and cartilage. Both constructs induced preaxial polydactyly, as well as truncation of the zeugopod. Both panels show forelimbs. Ectopic digits are indicated with an asterisk. Arrowheads indicate zeugopod abnormalities.

The role of the HLH region of dHAND

The above results suggested that neither the amino-terminal region, nor the carboxy terminal region, nor the basic region were required for dHAND to repattern the developing limb in our transgenic assay. To determine if the HLH motif was important for this activity, we deleted the this region within dHAND. As predicted, this mutant protein failed to coimmunoprecipitate with E12 or bind DNA (data not shown). Ten of ten *prx-ΔHLH* transgenic embryos displayed normal limb morphology (Fig. 24A). This mutant protein was expressed at levels comparable to the wild-type protein, as determined by western blotting and immunostaining of transfected COS cells with anti-Myc antibody (data not shown).

To further examine the role of the HLH region, we mutated a highly conserved phenylalanine to a proline in the first helix (mutant F119P). A similar mutation has been shown to abolish dimerization of other bHLH proteins (Chakraborty et al., 1991; Davis et al., 1990). Coimmunoprecipitation assays confirmed that this mutant was unable to dimerize with E12, but it was expressed at levels comparable to wild-type dHAND in transfected cells (data not shown). Three of three *prx-F119P* transgenic embryos exhibited completely normal limb morphology (Fig. 24B), confirming that a functional HLH motif is required for dHAND activity.

To test whether the HLH domain alone was sufficient to induce an ectopic ZPA, we expressed the dHAND bHLH under the control of *prx* regulatory sequences. Fifteen of fifteen *prx-dHAND-bHLH* transgenic embryos had normally patterned limbs (Fig. 24C). This suggests that although the HLH is required, it is not sufficient alone to induce an ectopic ZPA. This dHAND mutant protein was also expressed at levels equal to wild-type dHAND

FIGURE 24

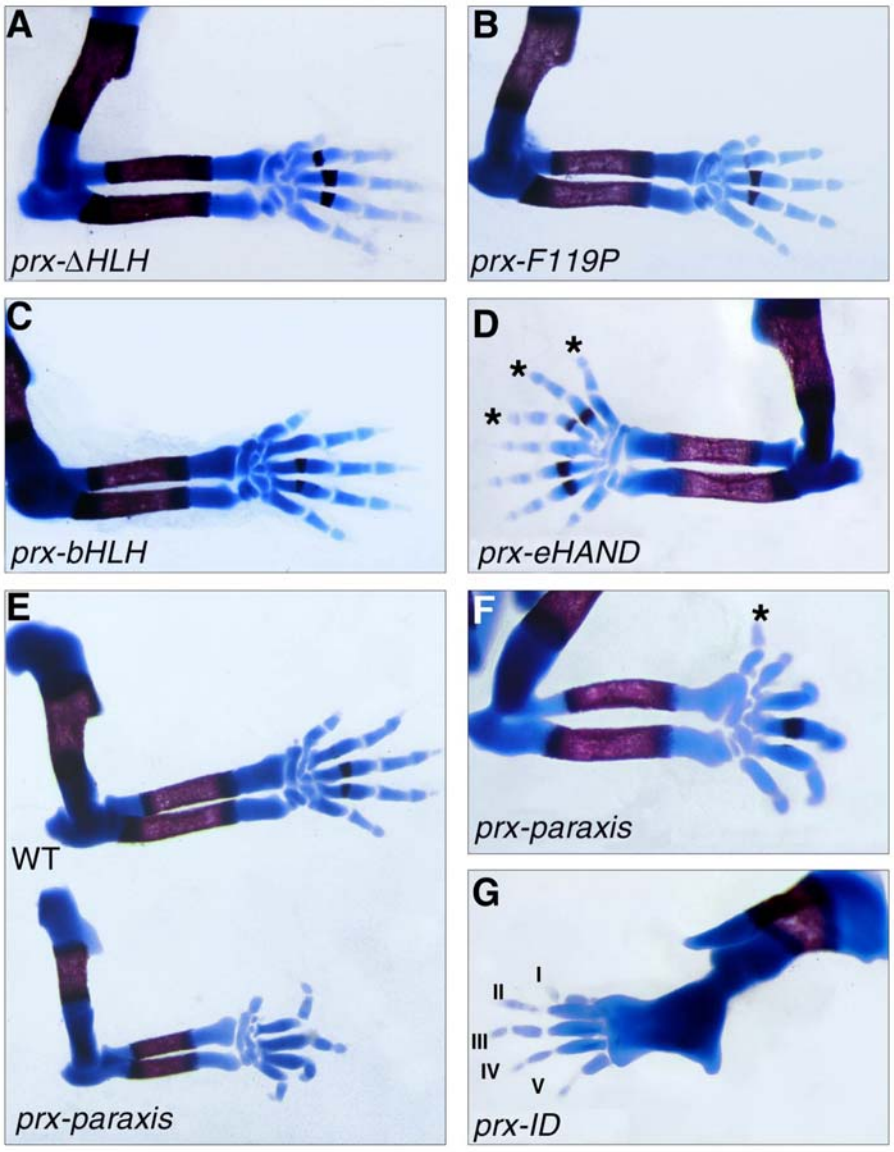

A
prx-ΔHLH
B
prx-F119P
C
prx-bHLH
D
*
*
*
prx-eHAND
E
WT
prx-paraxis
F
*
prx-paraxis
G
I
II
III
IV
V
prx-ID

Figure 24. Analysis of the role of the HLH region on limb patterning. F0 transgenic mouse embryos were sacrificed at E16.5 and limbs were stained for bone and cartilage. Transgenes are indicated in each panel. The *prx-ΔHLH* (A), *prx-F119P* (B), and *prx-bHLH* (C) transgenes had no effect on limb patterning. The *prx-eHAND* transgene (D) resulted in preaxial polydactyly indistinguishable from that resulting from *prx-dHAND*. The *prx-paraxis* transgene resulted in shortened zeugopodial bones (E) and only a single extra digit in 1 out of 11 transgenic embryos (F). Embryos harboring *prx-Id* transgenes displayed malformed zeugopodial structures and normally patterened digits that are labeled numerically (G). All panels show forelimbs. Ectopic digits are indicated with an asterisk.

in transfected COS cells, although we cannot rule out the possibility that it might be expressed at reduced levels in transgenic animals.

In order to determine if early limb patterning activity is uniquely encoded by the dHAND HLH region, we tested two related bHLH proteins in this assay. The dHAND and eHAND proteins differ at only one amino acid in the HLH region, but eHAND is not expressed in the developing limb. As shown in Fig. 24D, a *prx-eHAND* transgene was able to efficiently induce preaxial polydactyly that was indistinguishable from that of *prx-dHAND* transgenic embryos. Paraxis, which is expressed in the developing somites, but not in the early limb buds (Burgess et al., 1995), is 62% identical and 78% similar to dHAND within the HLH domain (data not shown). Transgenic embryos harboring a *prx-paraxis* transgene consistently showed shortened limbs (Fig. 24E). However, only a single biphalangeal ectopic digit was observed among 11 embryos harboring the *prx-paraxis* transgene (Fig. 24F). This suggests that although paraxis may be capable of recapitulating some aspects of dHAND function it is far less potent than dHAND in this assay. Together, these results suggest that *HAND* genes possess unique evolutionarily conserved functions that are not shared by other closely related bHLH proteins.

We also generated transgenic mice that expressed the inhibitory HLH protein Id under control of the *prx1* promoter. Id proteins, which contain an HLH motif, but lack a basic domain, dimerize with E-proteins, thereby inhibiting the activity of Class B bHLH proteins by sequestering their obligate dimerization partners (Benezra et al., 1990). Embryos harboring the *prx-Id* transgene showed normal patterning of the digits, but exhibited severe malformations of the zeugopods (Fig. 24G). These findings raise the possibility that the limb

patterning activity of dHAND does not require dimerization with E-proteins, although other interpretations are also possible.

Discussion

We and others previously demonstrated a critical role for dHAND in patterning the A-P axis of the limb and regulating *Shh* expression (Charité et al., 2000; Fernandez-Teran et al., 2000; Yelon et al., 2000). The results of this study extend those findings and lead to the unexpected conclusion that this activity of dHAND is mediated by the HLH region and involves a mechanism independent of direct DNA binding. The HLH region of eHAND, which shares high homology with dHAND, is also capable of repatterning the early limb, whereas the more distantly related bHLH protein, paraxis, lacks this activity. Our results suggest that the molecular mechanisms by which dHAND functions during development are more complicated than traditional models of bHLH protein function predict.

DNA-binding independent dHAND functions

Tissue-specific (class B) bHLH proteins are thought to act primarily as heterodimers with widely expressed E-proteins (Massari and Murre, 2000). Consistent with this notion, dHAND cooperates with E12 to bind DNA and activate transcription through the E-box consensus sequence in transfected cells. Mutations that abolish DNA binding or dimerization eliminate the transcriptional activity of dHAND in this assay. Similarly, deletion of the amino-terminal transcription activation domain of dHAND severely impairs its ability to activate transcription. These results demonstrate that dHAND can act as a typical class B bHLH transcription factor.

The finding that the basic region, which is required for DNA binding to E boxes, is not required to induce ectopic digits in transgenic mice is unanticipated and suggests that dHAND may act *in vivo* through mechanisms other than those observed in tissue culture reporter assays. In addition to mediating DNA binding, the basic regions of other bHLH proteins have been shown to participate in protein-protein interactions required for cell specification (Brennan et al., 1991; Davis et al., 1990). However, a dHAND mutant lacking the entire basic region was as active as the wild-type protein in transgenic mice, indicating that this region does not mediate association with transcriptional cofactors involved in this process.

Interestingly, the class B bHLH transcription factor SCL/Tal1 also exhibits DNA binding-independent activity *in vivo*. A mutant SCL protein lacking the basic region can rescue primitive hematopoiesis in SCL null embryoid bodies and in zebrafish lacking *cloche*, the SCL/Tal1 homolog (Porcher et al., 1999). Similarly, a point mutant SCL protein that lacks DNA binding activity can induce leukemia in transgenic mice (O'Neil et al., 2001). This suggests that many class B bHLH proteins, like dHAND and SCL/Tal1, may operate through direct DNA-binding independent mechanisms.

Although misexpression experiments must be interpreted with caution, the present results provide evidence that dHAND can regulate target genes in the early limb bud without binding directly to DNA. While this activity could be considered to represent some sort of nonphysiologic effect of ectopic expression, we do not believe this is the case for the following reasons. First, *dHAND* expression precedes the expression of *Shh* in the ZPA and encompasses the *Shh* expression domain at all stages of normal limb development. Second,

mouse and zebrafish *dHAND* mutant embryos fail to express *Shh* in the ZPA and exhibit aberrant limb patterning (Charité et al., 2000; Fernandez-Teran et al., 2000; te Welscher et al., 2002; Yelon et al., 2000). Third, altered limb patterning was only observed with ectopic dHAND and eHAND, but not with the related bHLH protein paraxis. This level of specificity would not be expected if this were a nonspecific effect of ectopic bHLH protein expression.

Potential roles for transcriptional activation in limb patterning by dHAND

Our results also demonstrate that either the amino- or the carboxy terminal regions of dHAND can be deleted without a loss of digit inducing activity. Since the amino terminal region of the protein contains a transcription activation domain, but the carboxy terminal region of the protein does not exhibit measurable transcriptional activity when fused to the GAL4 DNA-binding domain, this suggests that the intrinsic transcriptional activity of dHAND is not required for limb patterning. However, a limitation in interpreting these results is that the bHLH region of dHAND alone was devoid of patterning activity. We favor the interpretation that this small portion of the protein is unable to establish stable interactions in vivo with essential cofactors due to the possible absence of surrounding sequences to stabilize such interactions. However, because deletion of the carboxy terminal residues (Δ155-217) reduces the transcriptional activity of dHAND in tissue culture assays, we cannot rule out the possibility that a transcription activation domain is required for the limb patterning activity of dHAND and that the carboxy terminal region of the protein possesses transcriptional activity in vivo that is not detectable in GAL4-reporter assays.

Polydactyly in mice harboring the *prx-dHAND-VP16* transgene suggests that although intrinsic transcriptional activity of dHAND may not be required to induce ectopic digits, fusion of a potent transcriptional activation domain to dHAND, effectively creating a super-activating form of dHAND, also does not abrogate this activity. This finding is consistent with the notion that dHAND acts as part of a transcriptional activation complex. The requirement of dHAND for the expression of *Shh*, *Hoxd11*, *Hoxd12*, and *BMP2* in mice and fish also is consistent with dHAND acting as a transcriptional activator. However, te Welscher, et al (2002) have recently shown that dHAND is required for the repression of *Gli3* and *Alx4* in the posterior limb mesenchyme prior to *Shh* expression. This suggests that dHAND may have dual functions as both a transcriptional repressor and activator, although it remains possible that this apparent repressive activity of dHAND reflects the initial activation of a target gene that encodes a repressor of *Gli3* and *Alx4*. Because viral expression of dHAND in the anterior compartment of the limb does not repress endogenous *Gli3* or *Alx4* expression, but is sufficient to induce ectopic *Shh*, and *Gremlin*, it has been proposed that dHAND repression may require an as yet unknown bHLH partner expressed exclusively in the posterior compartment. However, it remains unclear which, if any, genes dysregulated in dHAND $^{-/-}$ limbs represent direct transcriptional targets. Considering that dHAND is required for the activation and repression of multiple genes at the same time and place in development, we favor the notion that at least some of these genes represent indirect transcriptional targets of dHAND.

Doublefoot mutant mice ectopically express *Ihh* along entire A-P dimension of the early limb bud, with resulting severe (6-8 digits) polydactyly (Yang et al., 1998). It has been

proposed that high levels of hedgehog signaling throughout the limb bud cause increased growth of the distal mesenchmye. This increased growth is reflected in formation of a greater number of ectopic digits. In addition, these ectopic digits display symmetric A-P identity, as a result of the disrupted hedgehog signaling gradient. We observed a similar phenotype in a subset of transgenic animals, most frequent noted in *prx-dHAND-VP16* embryos. Accordingly, we also detected a broad domain of ectopic *Shh* expression in *prx-dHAND-VP16* embryos. This suggests that dysregulated *Shh* signaling may be responsible for *Dbf*-like phenotypes observed in our transgenic embryos.

It is possible that increasing the transcriptional activation potential of dHAND by fusion to VP16 accounts for the broadened expression of *Shh*. At high levels of transactivation, that may also be dependent on transgene copy number and integration effects, dHAND may override the endogenous mechanisms that restrict ectopic *Shh* expression to the anterior margin. The higher frequency of *Dbf*-like phenotypes that result from expression of *dHAND-VP16*, which displays the highest transcriptional activity in tissue culture, supports this notion. It is interesting to note that the dependence of *Shh* expression on FGF-mediated signals from the AER does not seem to be affected, as ectopic *Shh* is always restricted to the distal mesenchyme underlying the AER (Laufer et al., 1994; Niswander et al., 1994). However, because we did not examine expression of *Shh* in transgenic embryos generated with other dHAND mutant proteins, it is also possible that the broad ectopic expression domain of *Shh* is unique to the *prx-dHAND-VP16* transgenic line.

Potential roles of the HLH region in limb patterning by dHAND

Mutations affecting the HLH domain of dHAND fail to induce ectopic digits in transgenic embryos. This is not unexpected, and suggests that protein interactions mediated by the HLH are required for activity of dHAND in vivo. However, overexpression of Id in the limb bud, which would be expected to sequester E-protein dimerization partners for dHAND, did not affect digit number or pattern. This finding raises the possibility that the dHAND HLH associates with partners other than E-proteins to regulate limb pattern.

Previous studies with eHAND have provided evidence that HAND proteins may indeed have multiple dimerization partners. Scott et al (2000) demonstrated that eHAND forms homodimers and, interestingly, E-proteins are downregulated in trophoblast tissue at a time when eHAND is believed to function. This provides circumstantial evidence that eHAND acts either as a homodimer or with other unknown partners in this tissue. In addition, the eHAND HLH domain is required for transcriptional repression and activation in tissue culture reporter assays, suggesting that the transcriptional functions of eHAND are modulated by the choice of HLH partners (Hollenberg et al., 1995; Scott et al., 2000). Two-hybrid and biochemical pull down experiments have also shown that eHAND and dHAND homodimerize as well as heterodimerize with one another as well as with members of the hairy-related (HRT) family of bHLH transcription factors (Firulli et al., 2000). In light of these studies, the work of te Welscher et al (2002), and the results presented here, it will be especially interesting to identify the transcriptional partners of dHAND in the developing limb and to determine how these interactions modulate dHAND transcriptional activity.

A possible role for dHAND in bone morphogenesis

Although we focused on digit number and morphology in our transgenic misexpression assay, we also noted severely malformed zeugopodial structures in embryos that expressed dHAND mutants lacking the basic and carboxy terminal regions (mutants Δbasic, RRR109-111EDE, and Δ155-217). Interestingly, these dHAND mutants exhibit reduced or no activity in transfection assays. Thus, it is possible that during zeugopod development dHAND binds DNA and requires protein-protein interactions mediated by the highly conserved carboxy terminal residues. Mutations in the DNA binding domain or the carboxy terminus may therefore generate dominant-negative dHAND molecules that interfere with the activity of the wild-type protein at this stage. The expression of *dHAND* in the perichondrium and hyperplastic chondrocytes of the ulna, coupled with the lack of forearm abnormalities in transgenic embryos expressing wild-type dHAND, support this notion and suggest that dHAND plays a role in bone maturation. Because we were interested primarily in early limb patterning by dHAND, a complete analysis of *dHAND* expression at all stages of bone development was not undertaken. However, based on our limited results, we favor the hypothesis that dHAND plays a role in the development of a subset of bones in the posterior zeugopod, rather than acting as a global regulator of bone growth and morphogenesis.

Because dHAND regulates expression of *Shh* during limb patterning, it is interesting to speculate that dHAND may also regulate *Indian hedgehog* expression during bone maturation. It will be interesting to address this and other issues in future experiments to determine the role of dHAND during bone development.

Functional redundancy of HAND proteins

The expression patterns of *dHAND* and *eHAND* overlap in several tissues during mouse and chick development, and studies with anti-sense oligonucleotides in chick embryos suggest that these two factors play redundant roles during cardiac development (Srivastava et al., 1995). In contrast, the zebrafish genome appears to encode only a single HAND protein, which is most similar to dHAND (Yelon et al., 2000). The zebrafish *hands off* mutation, which disrupts the *dHAND* ortholog, shows a more severe cardiac phenotype than the mouse *dHAND* knockout mutation (Yelon et al., 2000). Because no *eHAND* ortholog has been identified in zebrafish, this suggests that dHAND and eHAND play redundant roles in early heart development in higher vertebrates.

Although our transgenic results do not demonstrate genetic redundancy, they show that dHAND and eHAND proteins can function similarly when misexpressed in the anterior compartment of the developing limb. These findings suggest that dHAND and eHAND may act in a redundant fashion in other tissues during development, including heart, pharyngeal arch neural crest, and sympathetic neurons. Tissue-specific deletion of these genes should bypass global embryonic abnormalities and provide further insight into this issue.

Cumulatively, these and other studies suggest novel mechanisms of action of class B bHLH proteins. Although many bHLH factors, including dHAND and eHAND, act predictably according to these models in transfected cells, it appears that the mechanisms of action of these factors in vivo are more complex. Because of the critical roles played by bHLH transcription factors during embryonic development, determining the molecular

mechanisms by which these proteins operate should facilitate the identification of novel transcriptional targets that execute the developmental decisions controlled by bHLH proteins.

Methods

Cell Culture and Transfections

10T1/2 and COS cells were maintained in Dulbecco's Minimal Essential Medium with 10% fetal bovine serum. Cells were transfected using FuGENE 6 reagent (Roche) with 200 ng of reporter (L8G4-luciferase or L8E6-luciferase) and expression constructs (Hollenberg et al., 1995). The pcDNA3.1 vector without a cDNA insert was used to equalize the amount of DNA in each transfection. To control for transfection efficiency, 50ng of CMV-lacZ was included in all transfections. Cell extracts were prepared 48 hours after transfection and assayed for luciferase activity using the Promega Luciferase detection kit. Luciferase activities were normalized to β-galactosidase activity. Each experiment was performed in duplicate. Values from at least three independent experiments were averaged for each figure.

Coimmunoprecipitation and Western Blotting

Coimmunoprecipitation experiments were performed in COS-1 cells transiently transfected with 1μg of each expression vector. Immunoprecipitation reactions were performed as previously described (McKinsey et al., 2000). Briefly, cell extracts were harvested in 500μL of lysis buffer (1X PBS pH[7.4], 0.1% Triton X-100, 1mM EDTA, 1X Roche Complete protease inhibitor cocktail, 1mM PMSF). Following incubation on ice for 10 minutes, extracts were sonicated briefly and centrifuged at 4°C for 10 minutes. Monoclonal anti-FLAG antibody (Sigma) was added to the supernatant and rocked at 4°C for 1 hr. Protein G-

sepharose beads (Zymed) were added and rocking was continued for another hour. The beads were then washed 4 times by brief centrifugation, and rinsing in lysis buffer. Protein was released from the beads by addition of SDS-PAGE loading buffer and boiling for 5 minutes.

SDS-PAGE and western blotting was performed using standard techniques (McKinsey et al., 2000). A 1:1000 dilution of rabbit polyclonal anti-myc (Santa Cruz) antibody was used as a primary antibody, followed by a 1:10,000 dilution of donkey anti-rabbit horseradish peroxidase conjugated secondary antibody (Amersham).

Expression and transgene constructs

Mammalian expression vectors were generated from the dHAND cDNA by high-fidelity PCR using Pfu Turbo DNA polymerase (Stratagene) using standard techniques. Most cDNA fragments were cloned into a modified pcDNA3.1 vector containing an amino-terminal myc epitope tag (McKinsey et al., 2000). pSG424 and pM1 vectors used to generate GAL4-DBD fusion proteins, and the L8G5E1b reporter have been described elsewhere (Lemercier et al., 1998). All cDNAs generated by high-fidelity PCR were cloned into the *Eco*RI cloning site of pcDNA3.1 or pSG424. The mouse *prx1* promoter was used to direct the expression of dHAND cDNAs in transgenic mice. This promoter has been described previously (Martin and Olson, 2000). *Prx1* transgenes were generated by cloning wild-type or mutant dHAND cDNAs into a BglII site in the 5' untranslated region of the *prx1* gene. Transgenes also contained a 3' human growth hormone (hGH) polyadenylation signal. All dHAND

mutations were confirmed by DNA sequencing. Sequence of PCR primers is available upon request.

Gel mobility shift assays

Gel mobility shift assays were performed as described previously (McFadden et al., 2000). *In vitro* translated proteins were generated using the Promega TNT kit, and 3μL of lysate were used in each binding reaction. An oligonucleotide probe corresponding to the published eHAND/Thing-1 E-box was used (Hollenberg et al., 1995): Th-1A 5'-CTCGAGGGATCCAATGCATCTGGATCGGGGCA-3', Th-1B 5'-CTCGAGTGCCCCGATCCAGATGCAATGGATCC-3'. Oligonucleotides were annealed and radio-labeled as described (McFadden et al., 2000). DNA-protein complexes were resolved on a 6% nondenaturing polyacrylamide gel in 0.5XTBE and 2.5% glycerol.

Transgenic mice

Transgenic mice were generated as previously described (Cheng et al., 1993). F0 embryos were harvested by sacrificing the foster mother at the desired embryonic time point.. Embryos used for bone and cartilage staining were harvested, soaked in tap water overnight, skinned, eviscerated, and dehydrated for 48 hours before staining with Alcian blue and Alazarin red (Hogan, 1994).

The genotypes of transgenic embryos were determined by southern blotting. Yolk sacs were digested overnight in tail lysis buffer with 0.8μg/μL proteinase K, followed by a

second addition of proteinase K and further incubation for another 12-24 hours. Protein was extracted with phenol:chloroform (1:1), followed by chloroform. DNA was precipitated with an equal volume of isopropanol, followed by rinsing in 1ml of 70% ethanol. DNA was resuspended in 100μL deionized H_2O. 15μL of DNA was digested with EcoRV overnight. Southern blotting was performed using standard techniques with [^{32}P] radiolabeled probes corresponding to dHAND coding sequence used in transgene constructs.

In situ hybridization

In situ hybridization to embryo sections was performed as described previously, using the full length dHAND cDNA labeled with ^{35}S-UTP (Shelton et al., 2000). Whole mount *in situ* hybridization was performed as previously described (Riddle et al., 1993).

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Using genetically modified mice, we have investigated the functions of murine *HAND* genes during embryonic development in order to gain insights into the molecular pathways that control formation of the mammalian heart. Firstly, we have utilized transgenic mice to analyze the *cis*-acting regulatory sequences that govern *dHAND* expression in the embryonic right ventricle in hopes of identifying signals that establish chamber-restricted gene expression during cardiogenesis. Secondly, we have generated conditional knockout alleles of *eHAND* in order to investigate the roles of eHAND during embryonic heart development. Thirdly, we have misexpressed a series of *dHAND* mutant transgenes in limb buds to investigate the mechanisms by which dHAND regulates transcription of downstream target genes. Cumulatively, we hoped that these investigations would reveal both fundamental and clinically relevant mechanisms by which the heart and other three-dimensional organs form during embryonic development.

GATA transcription factors regulate expression of *dHAND* during heart development

Using transgenic mice, we have identified upstream transcriptional regulatory sequences that are sufficient to direct *dHAND* expression in the embryonic heart, and shown that activity of this enhancer depends on paired binding sites for GATA transcription factors. These results demonstrate that GATA factors are required for activity of this enhancer. However, one cannot extrapolate from these results that GATA factors are sufficient to generate right

ventricle-specific expression. In fact, evidence suggests otherwise: that GATA factors are insufficient to generate chamber specificity. First, deletion analysis of the dHAND enhancer demonstrates that other sequences are required for enhancer activity (Fig. 4). In addition, GATA factors are expressed throughout the myocardium (Arceci et al., 1993; Jiang and Evans, 1996; Laverriere et al., 1994; Morrisey et al., 1996; Morrisey et al., 1997). Therefore, GATA-dependency alone fails to explain how this enhancer is regulated in a chamber-specific manner during cardiac development.

It is possible that transcriptional activity of GATA factors is regulated in a chamber-specific manner, potentially through chamber-specific cofactor interactions or post translational modifications. It is more likely, however, based on deletion analysis, that an unidentified DNA-binding transcriptional regulator controls region-specific expression of the *dHAND* cardiac enhancer. The fact that *dHAND::lacZ* transgenes are initially expressed throughout the cardiac crescent, but are subsequently downregulated in the LV and inflow (future atria) during cardiac looping may suggest that chamber specificity is mediated by transcriptional repression. However, our analysis has so far failed to reveal a mutation or deletion that resulted in upregulation of reporter expression in the LV or inflow.

To date, a chamber-specific cofactor for GATA transcription factors has not been identified; as such, GATA factors, which are known to participate in the transcriptional regulation of numerous genes in the heart (Charron and Nemer, 1999), are more likely to regulate the level of expression or the initiation of transcriptional competence. One potential mechanism for this activity is the ability of GATA factors to recruit chromatin-remodeling complexes (Dai and Markham, 2001; Kakita et al., 1999; Wada et al., 2000). Binding of

GATA proteins to this enhancer may therefore relax chromatin structure and provide access for chamber-specific transcription factors.

In addition to the right ventricle-specific *dHAND* enhancer described in this work, several genes expressed throughout the heart have been shown to be regulated by chamber-specific transcriptional regulatory elements (Kelly et al., 1999; Lien et al., 1999; Molkentin et al., 2000; Ross et al., 1996). This suggests that transcription during cardiogenesis is achieved through the use of modular regulatory elements that control expression in a given cardiac chamber (Schwartz and Olson, 1999). This may reflect the evolution of the multi-chambered vertebrate heart from the primitive, linear tube of *Drosophlia* (Schwartz and Olson, 1999). Nonetheless, identifying the molecules that control chamber-specific expression remains an important avenue of research, as many congenital heart defects affect a particular chamber or region, and description of these pathways may allow the identification of novel disease-causing genes.

In some aspects, the *dHAND* cardiac enhancer is an ideal candidate for these analyses. This enhancer is expressed throughout the cardiac crescent and linear heart tube, and is downregulated in the LV and inflow during cardiac looping, the point at which the first morphologic manifestation of chamber identity occurs. Therefore, we believe that this enhancer responds to the signals that initiate chamber identity. However, experimentally, the dHAND cardiac enhancer is cumbersome. Deletion analysis has failed to narrow the critical region below 1.5kb, making in vitro assays and screens difficult. Therefore, it may be more practical to use one of several known chamber-specific enhancers with a more defined

critical region (Ross et al., 1996; Searcy et al., 1998) in these screens, and subsequently determine if similar mechanisms are utilized by other chamber-specific enhancers.

Several experimental approaches can be used to identify these pathways. If chamber-specific gene regulation is achieved at the level of DNA binding, gel-mobility shift assays can be used to identify chamber-dependent DNA-binding complexes. For example, protein extracts from the right ventricle would be expected to produce a complex that is not observed with extracts obtained from the left ventricle. The unique complex can the be identified using standard techniques, and the relevance of this factor can be tested in transgenic mice harboring an enhancer transgene that fails to bind the chamber-specific factor in vitro (For example see Charité et al., 2001).

Comparative genomics is a particularly powerful bioinformatic tool that will likely have a tremendous impact on the characterization of transcriptional regulatory elements. Many of the genetic pathways that control organ formation have been evolutionarily conserved (Fishman and Olson, 1997). As such, conservation of discreet enhancer elements has been used to identify DNA binding factors that act upon *cis*-regulatory elements (For examples see Rowitch et al., 1999; Xu et al., 1999). We have cloned and sequenced the 5' upstream region from chick *dHAND* and compared this sequence to the upstream flanking region from mouse and human *dHAND*. Although the elements controlling *dHAND* expression in the pharyngeal arches are highly conserved between mouse and chick (Charité et al., 2001), we have found no significant sequence conservation with the *dHAND* cardiac enhancer despite the fact that this region is well conserved between human and mouse (Fig. 5). However, as the completed genome sequences of many species become available,

comparative sequence analysis should help reveal the intricacies of transcriptional regulatory elements and the *trans*-acting factors acting upon them.

Myocardial *eHAND* expression is necessary for normal cardiac valve formation

Our analysis of *αMHC::Cre; eHAND*$^{lacZ/loxP}$ mice unexpectedly revealed that myocardial expression of *eHAND* is required for normal atrioventricular valve formation. We are currently investigating the cellular and molecular mechanisms that underlie these defects. Although our preliminary analyses suggest that no difference exists between wild-type and *eHAND* mutants, we are continuing to examine cell death and proliferation at early and late stages of endocardial cushion development. In addition, analysis of the expression of signaling molecules implicated in endocardial cushion morphogenesis is ongoing.

Myocardium at the atrioventricular and outflow junctions is uniquely capable of inducing endocardium to delaminate, undergo an epithelial to mesenchymal transformation, and invade the underlying extracellular matrix to form the endocardial cushions (Eisenberg and Markwald, 1995). Interestingly, *eHAND* expression overlaps with this region of myocardium (Cserjesi et al., 1995). Therefore, it is possible that hyperplasia of the endocardial cushions results from increased endocardial transformation in the absence of eHAND. Therefore, we are using a well described in vitro explant assay to determine if endocardium from eHAND mutants exhibits a difference in the frequency of this transformation (Brown et al., 1999; Eisenberg and Markwald, 1995)

Downstream effectors of eHAND

Cardiac-specific deletion of *eHAND* in mice is compatible with embryonic viability, and results in heart defects commonly observed in humans (Hoffman, 1995; Srivastava, 2001). Human and mouse *eHAND* are expressed in embryo-derived trophoblast tissue which contributes formation of the placenta (Cross et al., 1995; Cserjesi et al., 1995; Knofler et al., 2002). *eHAND* null mice die from placental abnormalities early in development, thus it would be expected that mutations in the human *eHAND* coding sequence would similarly result in early embryonic lethality, and are therefore unlikely to be responsible for human congenital heart disease (Firulli et al., 1998; Riley et al., 1998). However, downstream effectors of eHAND during heart development are potentially interesting disease candidates.

Using a candidate approach, we have identified the transcriptional coactivator *cited1* as a downstream target of eHAND, albeit it is currently unclear whether this regulation is direct or indirect. In addition, we have also undertaken an unbiased search for downstream targets of eHAND using gene chip technology. RNA from wild-type and mutant left ventricles at E9.5 has been isolated, and analysis is currently underway to identify differentially expressed genes. It will be an important avenue of future research to confirm differential expression and investigate the roles these genes play in the *eHAND* mutant phenotype as well as human congenital heart disease.

Genetic redundancy of mammalian *HAND* Genes

Interestingly, *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$*; dHAND*$^{+/-}$ embryos die from cardiac insufficiency at E10.5. This demonstrates that dosage-sensitive genetic redundancy exists between

mammalian *HAND* genes. In addition, these animals provide an opportunity to identify common targets of dHAND and eHAND during early heart formation. As an example, we have demonstrated that expression of *ANF* and *connexin 40* (*cx40*) is abolished in *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$*; dHAND*$^{+/-}$ embryos. Intriguingly, mice lacking the T-box transcription factor ,Tbx5, also fail to express *ANF* and *cx40* (Bruneau et al., 2001). Because *Tbx5* expression is not significantly downregulated in *Nkx2.5::Cre; eHAND*$^{loxP/lacZ}$*; dHAND*$^{+/-}$ *embryos*, it is interesting to speculate that Tbx5 and HAND proteins synergistically regulate transcription *ANF* and *cx40*. The promoter sequences of *ANF* and *cx40* have been characterized, and Tbx5 and Nkx2.5 have been shown to synergistically activate transcription through these elements (Bruneau et al., 2001; Hiroi et al., 2001). It will be important to determine if HAND proteins activate transcription of the *ANF* and *cx40* promoters cooperatively with Tbx5 and Nkx2.5, and identify the *cis*-acting sequences and protein-protein interactions that mediate transcriptional synergy.

In addition to placenta and heart, *eHAND* is expressed in the neural crest derived mesenchyme of the branchial arches, as well as neurons of the sympathetic ganglia (Cserjesi et al., 1995; Thomas et al., 1998a). *eHAND*^{loxP} alleles will also be used to address the role of eHAND in these tissues; therefore, we are acquiring *Wnt1::Cre* mice that have been used successfully to recombine floxed alleles throughout neural crest derived structures (Chai et al., 2000; Jiang et al., 2000).

Mechanisms of transcriptional regulation by dHAND

We initially hypothesized that dHAND regulates transcription of downstream target genes in a fashion similar to other well characterized class B bHLH proteins (Massari and Murre, 2000). Indeed, tissue culture assays using a multimerized E-box reporter gene have shown that dHAND binds directly to DNA as a heterodimer with the E protein E12, and is capable of activating transcription. Transcriptional activity of dHAND on this reporter requires amino acids 45-90, suggesting that this region acts as a transcriptional activation domain. However, although these results demonstrate the potential for dHAND to regulate transcription in this manner, they should be interpreted with caution since the reporter gene is controlled by a synthetic promoter. It remains to be demonstrated that dHAND utilizes similar mechanisms to activate transcription of a native promoter in cooperation with E proteins.

It is therefore particularly interesting that dHAND acts independently of direct DNA binding and the amino terminal transcriptional activation domain when misexpressed in the developing limb bud. These results suggest that dHAND may not operate in vivo as a traditional class B bHLH protein. Interestingly, several dHAND mutants that were capable of inducing ectopic digits also resulted in abnormal bone formation within the zeugopod. This may suggest that dHAND acts via multiple transcriptional mechanisms at different times and places during development. The work of others also supports this notion, as dHAND and eHAND have been shown to homodimerize and heterodimerize with other bHLH proteins (Firulli et al., 2000; Scott et al., 2000).

These data reveal that dHAND may act atypically in vivo; however, little insight into the molecular mechanisms by which dHAND acts independently of DNA binding currently exists. dHAND is required for the transcriptional activation of *Shh*, *Hoxd11*, *Hoxd12*, and *BMP2* during limb outgrowth (Charite et al., 2000; Fernandez-Teran et al., 2000; Yelon et al., 2000). Conversely, dHAND is also required for transcriptional repression of *Gli3* and *Alx4* (te Welscher et al., 2002). Therefore, it is possible that dHAND acts as both a transcriptional activator and transcriptional repressor during limb development. However, because dHAND is required for both the activation and repression of multiple genes at the same time and place during development, we believe it is more reasonable to propose that dHAND acts as either an activator or repressor during limb development, and that some of these targets are indirectly regulated by dHAND.

Alternatively, dHAND may act as an indirect transcriptional regulator. For example, dHAND could inhibit DNA binding of a transcriptional repressor to a target promoter, which may include the regulatory elements of *Shh*, *Hoxd11*, *Hoxd12*, or *BMP2*, via a protein-protein interaction. In fact, none of our data contradict this possibility. Therefore, it will be critical to identify the protein partners that cooperate with dHAND during limb development, as it appears promoter specificity is not encoded by dHAND, but by the unknown protein(s) with which it interacts. It is interesting to speculate on the expression pattern of this unknown factor: because dHAND is sufficient to induce ectopic *Shh* when misexpressed in the anterior margin of the limb, this factor should be expressed throughout, or at both the anterior and posterior margins, of the limb mesenchyme. Yeast two-hybrid screening with

bait libraries generated from early limb buds is one possible method to identify these partners.

Concluding remarks

By and large the efforts described here have been successful: each independent study has contributed to an ever-expanding body of knowledge about how embryos develop complex organs during development. However, many questions remain unanswered, and it has been to my pleasure that even more have been raised throughout the course of this work.

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