A GATA-dependent right ventricular enhancer controls *dHAND* transcription in the developing heart

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SUMMARY

Heart formation in vertebrates is believed to occur in a segmental fashion, with discreet populations of cardiac progenitors giving rise to different chambers of the heart. However, the mechanisms involved in specification of different chamber lineages are unclear. The basic helix-loop-helix transcription factor dHAND is expressed in cardiac precursors throughout the cardiac crescent and the linear heart tube, before becoming restricted to the right ventricular chamber at the onset of looping morphogenesis. dHAND is also expressed in the branchial arch neural crest, which contributes to craniofacial structures and the aortic arch arteries. Using a series of *dHAND-lacZ* reporter genes in transgenic mice, we show that cardiac and neural crest expression of *dHAND* are controlled by separate upstream enhancers and we describe a composite cardiac-specific enhancer that directs *lacZ* expression in a pattern that mimics that of the endogenous *dHAND* gene throughout heart development. Deletion analysis reduced

this enhancer to a 1.5 kb region and identified subregions responsible for expression in the right ventricle and cardiac outflow tract. Comparison of mouse regulatory elements required for right ventricular expression to the human *dHAND* upstream sequence revealed two conserved consensus sites for binding of GATA transcription factors. Mutation of these sites abolished transgene expression in the right ventricle, identifying *dHAND* as a direct transcriptional target of GATA factors during right ventricle development. Since GATA factors are not chamber-restricted, these findings suggest the existence of positive and/or negative coregulators that cooperate with GATA factors to control right ventricular-specific gene expression in the developing heart.

Key words: Heart formation, dHAND, GATA transcription factor, mouse

INTRODUCTION

Classical embryology and cell fate mapping experiments have defined the morphological events involved in formation of the vertebrate heart (reviewed by Olson and Srivastava, 1996; Fishman and Chien, 1997). 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 (reviewed by 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 (reviewed in Massari and Murre, 2000). The related bHLH proteins, dHAND and eHAND, also known as Hand2/Thing2 and Hand1/Thing1, respectively, show dynamic and complementary expression patterns in the developing heart (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995; Srivastava 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., 1998). 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., 1998). 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 mutations 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), 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 (reviewed in 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 the GATA family is regulated in a chamber-specific manner during heart development.

MATERIALS AND 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-β-gal reporter.

One human *dHAND* genomic clone was isolated from a λ-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 (J. Charité and E. Olson, unpublished data) was blunt-cloned into the *Hin*dIII 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′-GATCTGTATTTCCCCTTAAAAA-GAGACTG-3′ and Primer 2, 5′-GAGGAGGGATCC-GTACCCTG-AATCAG-3′. Construct 9: Primer 3, 5′-GAGGAGGGATCCGATC-TATGGTGCTTG-3′, and Primer 2. Construct 11: Primer 4, 5′-CTAGTGCTTCATTCTTCCATAA-GTCTCAGG-3′, and Primer 5, 5′-TCTGTGATGACGCTGG-TTGTGGGC-3′. Construct 12: Primer 1 and Primer 6, 5′-CTTCATGGATCTTAGGAACTCAAAC-CC-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 *Sal*I to remove vector sequences, and purified for injection using a QiaQuick spin column protocol (QIAGEN). Concentrated DNA was eluted into 10 mM Tris buffer, pH 7.4. 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 β-galactosidase activity as described by 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 μm, 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 *GATA4* (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′-TCGAGGTGAGGATG<u>CTATC</u>TGGAGCT-3′;
−3039B, 5′-TCGAGAGCTCC<u>AGATAG</u>CATCCTCAC-3′;
−3140A, 5′-TCGAGGTAATTAAC<u>TGATAA</u>TGGTGC-3′;
−3140B, 5′-TCGAGGCACCA<u>TTATCA</u>GTTAATTAC-3′.

Mutant oligonucleotides were also synthesized as follows:

mut3140A, 5′-TCGAGGTAATTAACCCGGGATGGTGC-3′;
mut3140B, 5′-TCGAGGCACCATCCCGGGTTAATTAC-3′;
mut3039A, 5′-TCGAGGTGAGGATGGGCCCTGGAGCT-3′;
mut3039B, 5′-TCGAGAGCTCCAGGGCCCATCCTCAC-3′.

Oligonucleotide pairs were resuspended, boiled for 10 minutes, 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-GATA4 (Lu et al., 1999) or empty vector. Whole cell extracts were isolated as previously described by Brockman et al. (1995). DNA binding assays were performed as described by Scott et al. (1994). 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× TBE.

Site-directed mutagenesis

Conserved GATA sites at −3039 and −3140 were mutated to *Apa*I and *Sma*I 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′-CCCCTGGATTGTAGTAATTAACCCGGG-ATGGTGCTGTCAGTG-3′; GATAmut3140B, 5′-CACTGACA-GCACCATCCCGGGTTAATTACTACAATCCAGGGG-3′; GATA-mut3039A, 5′-CACTTGTGAGGATGGGCCCTGGAGCTACGAA-GCC-3′; GATAmut3039B, 5′-GGCTTCGTAGCTCCAGGGCCC-ATCCTCACAAGTG-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.

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., 1998). 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 (Charité 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 enhancer to the region between −8.0 and −7.75 kb (J. Charité and E. Olson, unpublished data). 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

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 on the left, and the corresponding expression pattern is summarized on the right. The far-right column indicates the fraction of F_0 transgenic embryos showing heart expression that also showed branchial arch expression, except for construct 2. For that construct (indicated by ‡), which lacks the branchial arch enhancer, we observed X-gal staining in the hearts of 5 out of 9 stable transgenic lines and in one F_0 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 F_0 transgenics. Constructs 1-7 contained the indicated
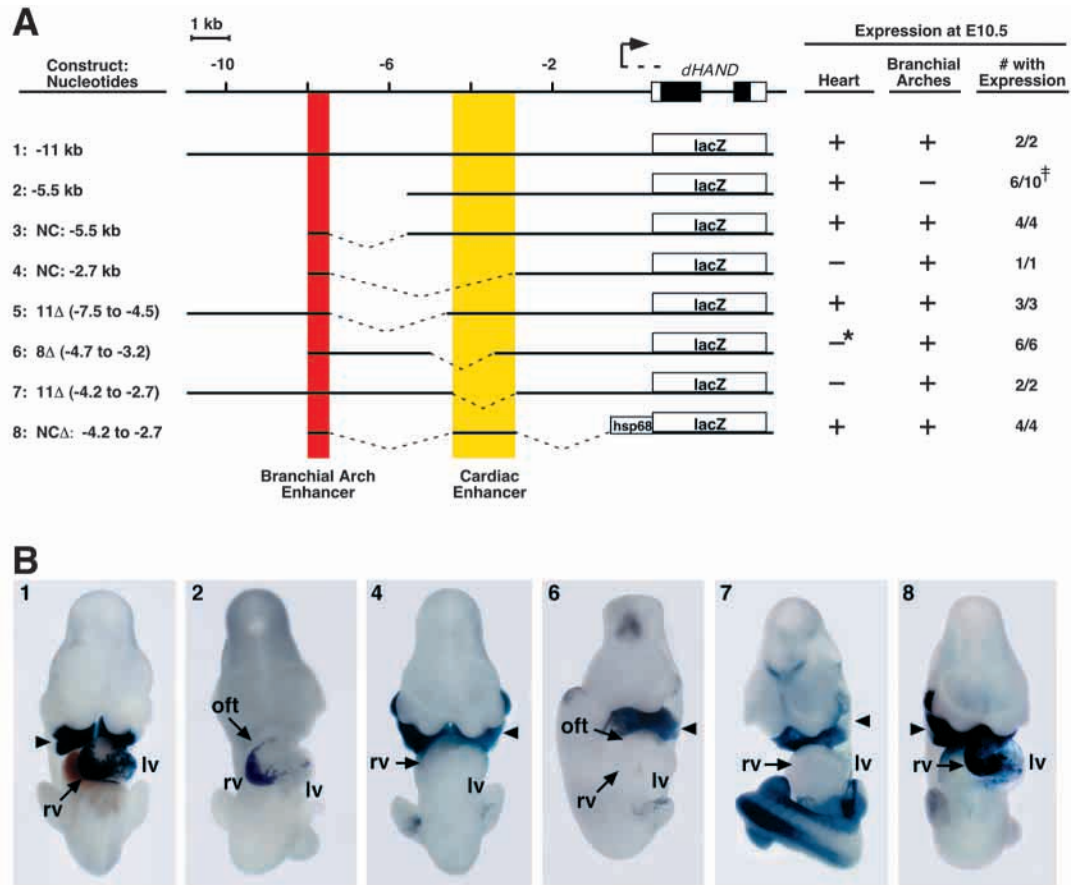
dHAND upstream region linked to the promoterless AUGβgal reporter. Construct 1, which directed expression in the first and second branchial arches and heart, contained 11 kb of upstream sequence. Cardiac expression was primarily noted at highest levels in the future RV and OFT. Because the embryo shown expressed *lacZ* at a very high level, low levels of transgene expression can also be seen in the future LV. Construct 2, containing 5.5 kb of upstream sequence, was expressed specifically in the heart. Construct 3 (not shown), which contained the branchial arch enhancer (−8/−7.75 kb) and the sequence in construct 2, produced *lacZ* expression in the branchial arches and heart. Construct 4, which fused the branchial arch enhancer to –2.7 kb, was active in the branchial arches, but not in the heart. Construct 5 (not shown) contained the 11 kb upstream region with a deletion from –7.5 to –4.5 kb, and directed branchial arch and cardiac expression. Construct 6 contained the 8 kb upstream region with a deletion from –4.7 to –3.2 kb, and directed expression in the branchial arches and in only a few cells in the RV. Construct 7 contained the 11 kb upstream region with a 1.5 kb deletion from –4.2 to –2.7 kb, and was expressed only in branchial arches. Construct 8 fused the neural crest enhancer and the region from –4.2 to –2.7 kb to the *hsp68lacZ* reporter. This construct directed branchial arch and cardiac expression. 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.

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., 1998). At E8.0, *lacZ* was
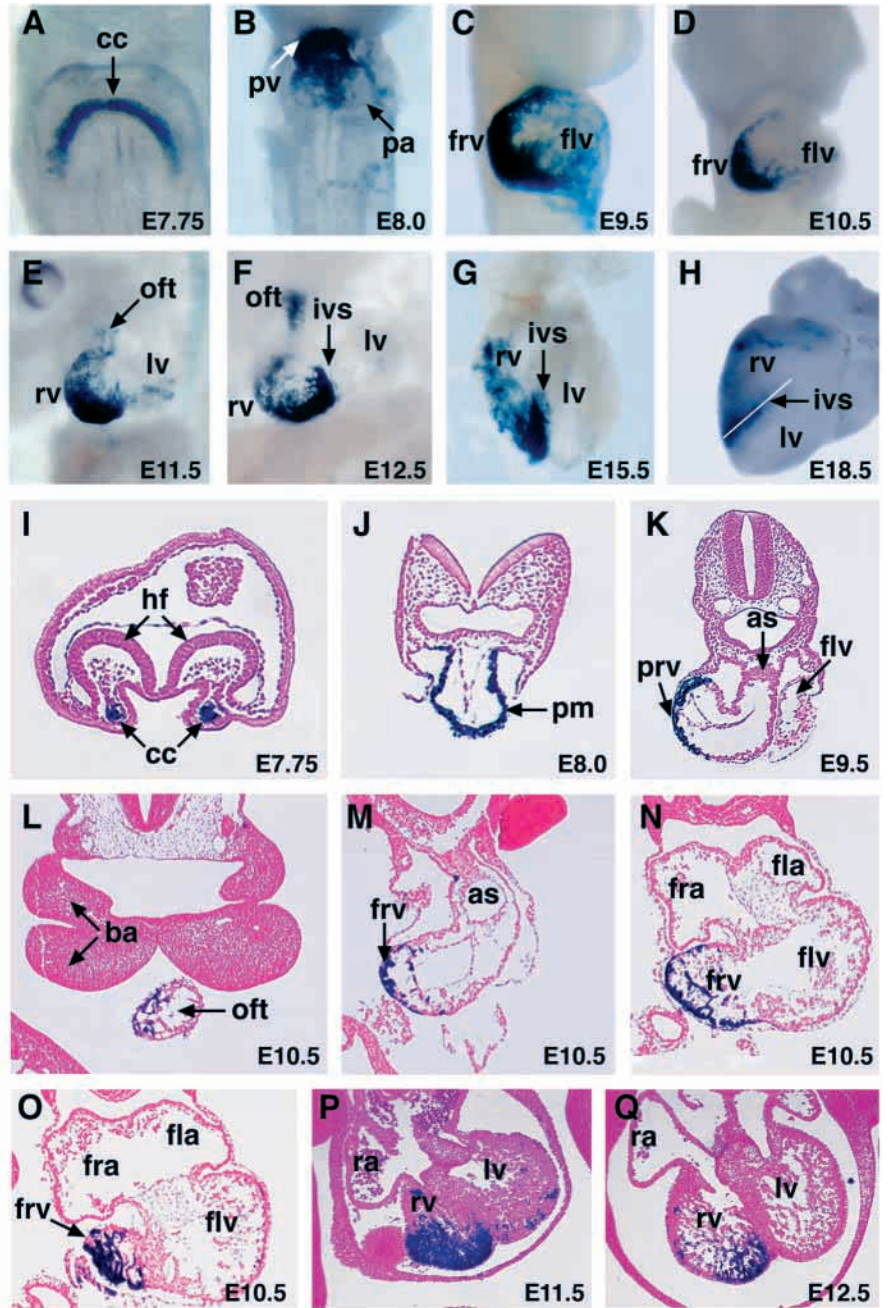
Fig. 2. Expression of the *dHAND* cardiac enhancer throughout embryonic development. Embryos from stable transgenic line 165 harboring construct 2 were analyzed at various times during mouse embryogenesis. (A-H) Whole-mount photographs of embryos or isolated hearts from E7.75-E18.5. The embryo in D is 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 interventricular 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.

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 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
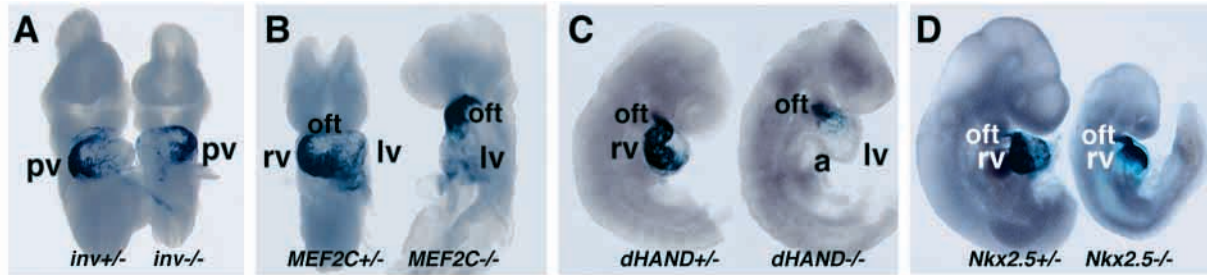
Fig. 3. Altered expression of the *dHAND* cardiac enhancer in mice with aberrant ventricular development. Construct 2 (Fig. 1) was introduced by breeding into the indicated mutant backgrounds and embryos were stained for *lacZ* expression. (A) Frontal views of *inv*$^{+/-}$ and *inv*$^{-/-}$ embryos 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.

expression detected at later time points were at 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., 1998). 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., 1998). 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* expression was 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.

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 β-gal-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 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*

Fig. 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 on 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 did not produce any cardiac gene expression in transgenic embryos. (D) 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, resulted in identical patterns of expression, with *lacZ* detected in a few cells of the outflow tract (oft). (E) Construct 12 contained the region from –4.2 to –3.2 kb, and directed expression in the OFT. 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.
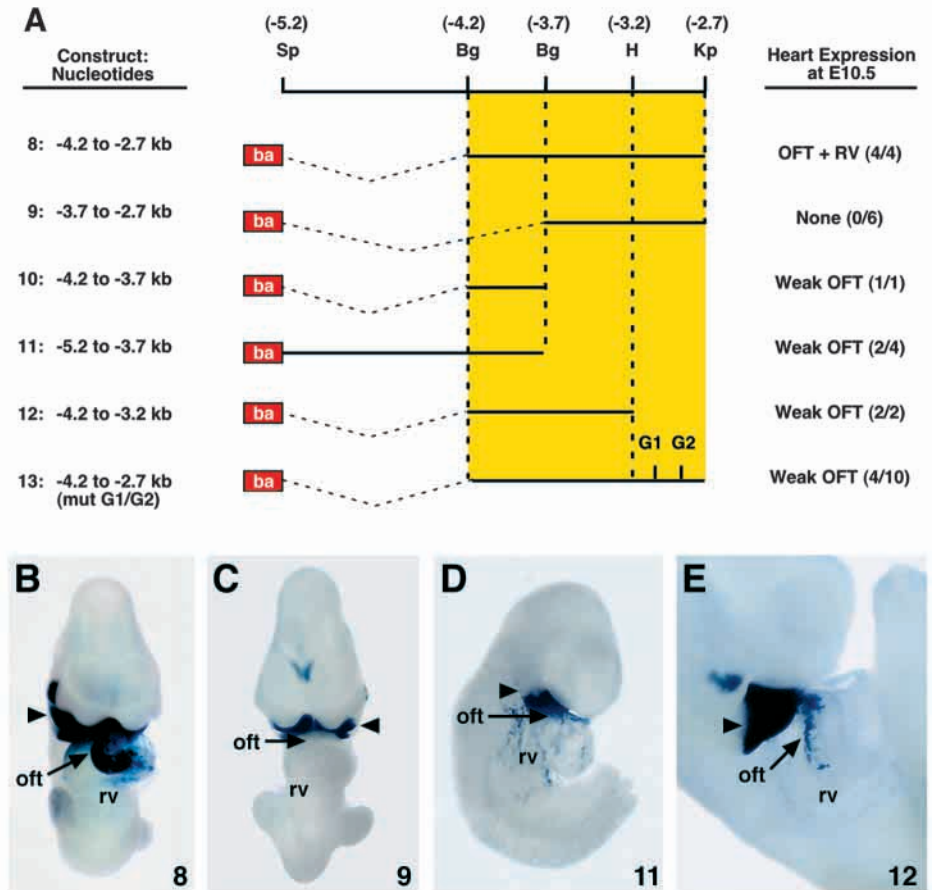
genomic phage clone and compared the upstream sequences of mouse and human *dHAND*. This alignment revealed a high degree of homology to the corresponding mouse sequence throughout the upstream region. The degree of homology was not significantly different within the putative cardiac enhancer compared 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.

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 GATA4 expression vector, and whole cell extracts were used in the assay. As shown in Fig. 6, GATA4 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 GATA4 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 F0 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 GATA4 was uniquely required for enhancer activity in vivo, we crossed the 5.5 kb enhancer transgenic line (construct 2) into a *GATA4* mutant background (Molkentin et al., 1997). Analysis of *lacZ* expression in *GATA4* null embryos showed that transgene expression was maintained in the absence of GATA4 (Fig. 7C) *GATA4* mutant embryos exhibit cardia bifida due to a defect in ventral morphogenesis of the embryo (Molkentin et al., 1997; Kuo 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 *GATA4* mutant embryos suggests that GATA5 or GATA6, which are expressed in an overlapping pattern with GATA4 during cardiac development, can substitute for GATA4 to activate the *dHAND* enhancer.

Fig. 5. Alignment of the *dHAND* 5′ flanking region from mouse and human. Bestfit alignment of the region from –4.2 to –2.7 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.

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., 1998). 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 (J. Charité and E. Olson, unpublished data).

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 (reviewed by 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

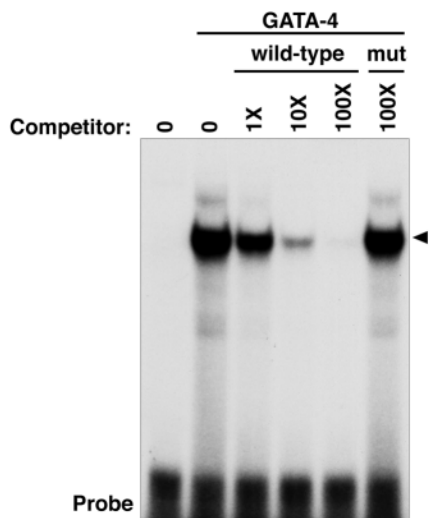The *dHAND* cardiac enhancer contains two conserved

Fig. 6. Binding of GATA4 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 GATA4-transfected cells (arrowhead). The far left lane contained extract from untransfected COS-1 cells. In addition, unlabeled wild-type oligonucleotide efficiently competed for DNA binding, whereas unlabeled mutant oligonucleotide did not compete.

Fig. 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 *GATA4* 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.

consensus binding sites for GATA factors, which bind GATA4 with high affinity and are required for enhancer activity. GATA4/5/6 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, 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 GATA4 expression blocks cardiac muscle differentiation, and that overexpression of GATA4 enhances cardiogenesis (Grepin et al., 1997, 1995). *GATA4* 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 *GATA4* null embryos, suggesting that these factors may act redundantly. This may explain the finding that the *dHAND* cardiac enhancer was active in *GATA4* 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 (Srivastava et al., 1997; Lin 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
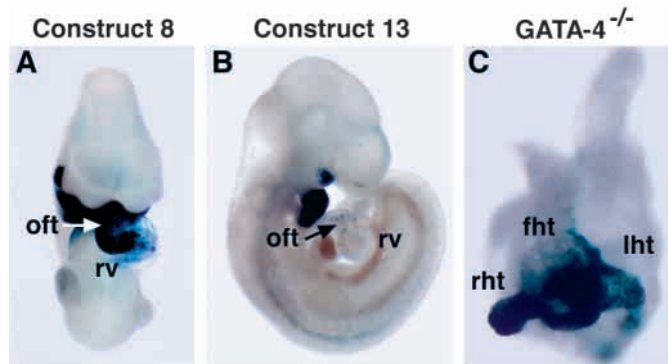
cardiac genes lacking MEF2 sites, by association with GATA factors (Morin et al., 2000). Thus, it is conceivable that MEF2C might regulate cardiac expression of *dHAND* via the essential GATA sites in the *dHAND* cardiac enhancer.

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., 1999). Similarly, the *CARP* promoter contains a 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 *GATA6* in the RV (Molkentin et al., 2000; Davis 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, GATA4 has been shown to cooperate with Nkx2.5 and MEF2 to activate cardiac target genes (Durocher et al., 1997; Sepulveda et al., 1998; Morin et al., 2000). Transcriptional activity of GATA factors has also been shown to be inhibited by the multitype zinc-finger protein FOG2, expressed predominantly in the heart (Tevosian et al., 1999; Sevensson et al., 1999; Lu 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 in each cardiac chamber.

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