



Human *eHAND*, but not *dHAND*, is Down-regulated in Cardiomyopathies

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A. NATARAJAN, H. YAMAGISHI, F. AHMAD, D. LI, R. ROBERTS, R. MATSUOKA, S. HILL AND D. SRIVASTAVA. Human *eHAND*, but not *dHAND*, is Down-regulated in Cardiomyopathies. *Journal of Molecular and Cellular Cardiology* (2001) 33, 1607–1614. The progression of cardiomyopathy to congestive heart failure is often associated with the expression of fetal cardiac-specific genes. In mice, the basic helix-loop-helix transcription factors, *dHAND* and *eHAND*, are expressed in a cardiac chamber-specific fashion and are essential for fetal cardiac development, but are down-regulated in the adult. Their expression in specific chambers of healthy and diseased human hearts has not been studied previously. Human *dHAND* and *eHAND* were mapped to human chromosomes 4q33 and 5q33, respectively, by fluorescent *in situ* hybridization. RNA from the four chambers of healthy human adult hearts, and from hearts of patients with several forms of cardiomyopathy, was obtained and assayed for *dHAND* and *eHAND* expression. Unlike in mice, *dHAND* expression was observed in all four chambers of the healthy human adult heart, but was diminished in the right atrium. In contrast, *eHAND* was expressed in the right and left ventricles, but was downregulated in both atrial chambers. We examined tissue from 15 human cardiomyopathic hearts obtained during cardiac transplantation or by endomyocardial biopsy for alterations in *HAND* gene expression. *dHAND* expression was unchanged in all forms of cardiomyopathy tested. However, cardiac expression of *eHAND* was severely down-regulated in six of six patients with ischemic cardiomyopathy and six of six patients with dilated cardiomyopathy. This study demonstrates that human *dHAND* and *eHAND* have unique spatial patterns of expression within human cardiac chambers. Downregulation of *eHAND* in ischemic and dilated cardiomyopathy suggests a correlation between *eHAND* dysregulation and the evolution of a subset of cardiomyopathies. © 2001 Academic Press

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Introduction

Cardiomyopathy is the result of abnormal systolic and diastolic function of the heart and leads to premature death from congestive heart failure (CHF) and cardiac arrhythmias. Approximately four million people in the United States suffer from CHF, resulting in four hundred thousand deaths annually.¹ Ischemic heart disease and idiopathic dilated cardiomyopathy, both of which lead to CHF,

are the two most common indications for heart transplantation in adults over the age of forty.² Regardless of the inciting event, the course of ischemic and dilated cardiomyopathy is characterized by remodeling of the ventricular muscle. The initial hypertrophic compensatory response fails to meet the circulatory requirements leading to progressive dilation and thinning of the myocardium. In contrast, primary hypertrophic cardiomyopathy, the result of mutations in sarcomeric proteins,³ and

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infiltrative cardiomyopathies⁴ less commonly lead to cardiac dilation. The remarkable similarities in the clinical course, histopathologic findings and hemodynamic abnormalities in patients with ischemic or dilated cardiomyopathy suggest that common molecular and cellular pathways regulating cardiomyocytes contribute to the progression of CHF. Accordingly, numerous overlapping signaling pathways have been implicated in the onset and development of cardiomyopathy.⁵⁻⁸

dHAND and *eHAND* are basic helix-loop-helix (bHLH) transcription factors that are necessary for heart development after the stage of cardiac looping in chick embryos, where they appear to have overlapping functions.^{9,10} Their expression in the mouse is chamber-specific during development, with *dHAND* selectively expressed in the right ventricle^{11,12} and *eHAND* in the left ventricle.^{12,13} The expression of *dHAND* and *eHAND* is specific to the pulmonary and systemic ventricular chambers regardless of right-left sidedness¹² but becomes diminished in late gestation and adult mouse hearts.^{9,10} *dHAND*-null mice demonstrate hypoplasia of the right ventricle from excessive programmed cell death.^{11,14} *dHAND* mutants also have excessive programmed cell death of the pharyngeal arches¹⁵ and aortic arch vessels¹⁶ and subsequently develop congestive heart failure and die by embryonic day 10.5. Mice lacking *eHAND* die early from placental defects precluding precise analysis of its role in cardiogenesis, however *eHAND* also appears necessary for normal cardiogenesis.^{17,18}

The role of the *HAND* genes in normal and abnormal human adult hearts has not been studied. However, alterations in the expression of several other fetal cardiac-specific genes have been demonstrated in cardiomyopathy.¹⁹ For example, cardiac expression of atrial natriuretic peptide,²⁰ brain natriuretic peptide,²¹ cardiac α -actin and β -myosin heavy chain (β -MHC) is upregulated in models of cardiomyopathy.²² The re-expression of fetal cardiac-specific genes in the adult diseased heart suggests that they may have a role in the evolution of, or response to, cardiac dysfunction.

Here, we have isolated human genomic clones of *dHAND* and *eHAND* and examined their mRNA expression in normal human hearts and in human cardiomyopathies. The cloning of human *dHAND* and *eHAND* cDNAs has been reported.^{23,24} Mapping by fluorescent *in situ* hybridization (FISH) confirmed radiation hybrid mapping results placing *dHAND* on 4q33 and *eHAND* on 5q33. Further, chamber-specific analysis of *HAND* gene expression revealed that human *dHAND* was expressed in all chambers of the normal adult heart, while *eHAND* expression

was relatively ventricle-specific. We searched for alterations in *HAND* gene expression in human cardiomyopathies compared to normal hearts. Unexpectedly, while *dHAND* expression was unchanged in all types of cardiomyopathies examined, *eHAND* expression was virtually undetectable in the hearts of patients with ischemic or dilated cardiomyopathy. *eHAND* expression was not altered in hearts of a few patients with hypertrophic or sarcoid cardiomyopathy. These results provide a molecular marker for human dilated or ischemic cardiomyopathies and suggest that alterations in *eHAND* expression may play a role in the evolution of some types of human cardiomyopathy.

Methods

Cloning of human *dHAND* and *eHAND*

A lambda gt11 human genomic library (CLONTECH) was screened using a ³²P-labelled human EST clone with high homology to mouse *dHAND*. Two $\times 10^5$ phage colonies were screened under conditions of low stringency. Briefly, filters were hybridized to the radiolabelled probe at 42°C overnight, followed by rinsing in 2 \times SSC, 0.1% SDS for 10 min at 50°C and 2 \times SSC, 0.1% SDS for 15 min at 65°C. Positive colonies were plaque-purified and genomic DNA subcloned into plasmid vectors. Clones were sequenced with *dHAND* or *eHAND*-specific primers on both strands by automated sequencing. Intron and exon borders were determined by comparing cDNA and genomic sequence.

Chromosomal mapping of human *dHAND* and *eHAND*

Human metaphase chromosome slides were prepared from Epstein-Barr virus-transformed lymphoblastoid cell lines or peripheral blood. Slides were hybridized with a 10 kb genomic clone encompassing *dHAND* or *eHAND* that had been obtained by screening a human genomic library (Clontech) as described above. FISH was performed overnight at 37°C and the slides washed with 50% formamide/2 \times SSC at 37°C for 10 min and 2 \times SSC for 10 min at room temperature. Fluorescent signals were detected using a Carl Zeiss fluorescence microscope. Known markers of 4q33 and 5q33 were co-hybridized and used to confirm chromosome localization.

RT-PCR of human heart tissue

Human tissue was obtained after informed consent, approved by an institutional review board, and in compliance with all institutional guidelines. Cardiac tissue was obtained from the right atrium, left atrium, right ventricle and left ventricle of healthy hearts from three young, adult male accident victims. Total RNA was extracted using Trizol and mRNA isolated using Qiagen Oligotex columns and reverse transcribed. PCR reactions were performed using the following primers: human *dHAND*: upper 5'-ACCCGCCGACACCAAACCT-3'; lower: 5'-GGTCTCCTCCTCCTCCTT-3'; human *eHAND*: upper 5'-GGAAAGGCTCAGGACCCAAGA-3'; lower 5'-GC-CATCCGCCCTTCTTGAGTTC-3'. PCR was performed at 94°C for 5 min; 26 cycles of 94°C for 30 s, 60°C for 3 min, 72°C for 3 min; and extension at 72°C for 7 min. PCR products were analysed between 20 and 32 cycles of amplification, in the linear range, by agarose gel electrophoresis and Southern blot with ³²P-labelled human *dHAND* or human *eHAND*. The integrity and amount of RNA was assayed by RT-PCR of glyceraldehyde 3-phosphate dehydrogenase (G3PDH). Non-reverse transcribed mRNA from the same tissues was used as negative control of the RT reaction in all samples. Reverse transcribed RNA from normal hearts was used as template for comparison of *dHAND* or *eHAND* expression in cardiomyopathies in the same experiment.

Cardiac ventricle tissue was obtained from hearts of adults of varying age and sex with CHF due to ischemic ($n = 6$), dilated ($n = 6$), hypertrophic ($n = 2$) or sarcoid cardiomyopathy ($n = 1$) at the time of heart transplant (right ventricle) or by endomyocardial biopsy of the right ventricular septal wall. Total RNA was isolated from the heart tissue using Trizol. RT-PCR reactions for *dHAND* and *eHAND* expression were performed as described above.

Results

Chromosomal mapping of *dHAND* and *eHAND*

To determine whether human *dHAND* or *eHAND* may be involved in heart defects associated with chromosomal anomalies, a human genomic library was screened under conditions of low stringency with the human *dHAND* cDNA. Of nine clones sharing homology to *dHAND*, four clones had exact sequence match with human *dHAND* and five were identical to human *eHAND*. No other *HAND* genes were identified in this genomic screen, suggesting an absence of other *HAND*-related family members

in humans. Sequence comparison of the human *dHAND* and *eHAND* cDNA with the corresponding genomic clones revealed the presence of a single intron in each gene. This intron began at identical amino-acid residues in *dHAND* and *eHAND*, suggesting a close ancestral relationship between the two genes [Fig. 1(A)].

Chromosomal positions of human *dHAND* and *eHAND* were previously determined by radiation hybrid mapping.^{23,24} To provide an alternate method to determine their precise chromosomal locations, we isolated 10 kb genomic fragments of *dHAND* or *eHAND* and fluorescently labelled them for fluorescent *in situ* hybridization analysis. We found that *dHAND* mapped to human chromosome 4q33 [Fig. 1(B)]. A known 4q33-specific marker was co-hybridized for more precise localization. Deletions encompassing 4q33 in humans have been reported and are frequently associated with congenital heart defects and cleft lip/palate, among other anomalies.²⁵ *eHAND* mapped to human chromosome 5q33, although no known human diseases map to 5q33 [Fig. 1(C)]. The position of *eHAND* was confirmed by co-hybridizing with a labelled 5q33-specific probe.

Chamber-specific expression of the *HAND* genes

Although *dHAND* and *eHAND* expression was not detected in the adult mouse,^{9,10} previous reports indicated expression in human adult.^{23,24,26} The complementary expression of *dHAND* and *eHAND* in the right and left ventricles of the embryonic mouse heart,^{11,12} respectively, led us to investigate the chamber-specific expression pattern of *dHAND* and *eHAND* in the human adult heart. Human *dHAND* expression was observed in the right atrium, right ventricle, left atrium and left ventricle of three normal adult hearts, represented in Figure 2. However, the expression in the right atrium was considerably less than that in the other chambers of all three specimens studied. This pattern of expression differed from that seen in the embryonic mouse heart,^{11,12} where *dHAND* was expressed selectively in the right ventricle, and down-regulated in the left ventricle and both atria. In contrast, human *eHAND* was expressed in the right and left ventricles, but was barely detectable in the atria of the same three normal adult hearts (Fig. 2). This differed from the selective left ventricular expression of *eHAND* in the embryonic mouse.^{11,12} The expression of *eHAND* predominantly in the ventricles provides a relatively ventricle-specific marker for human hearts.

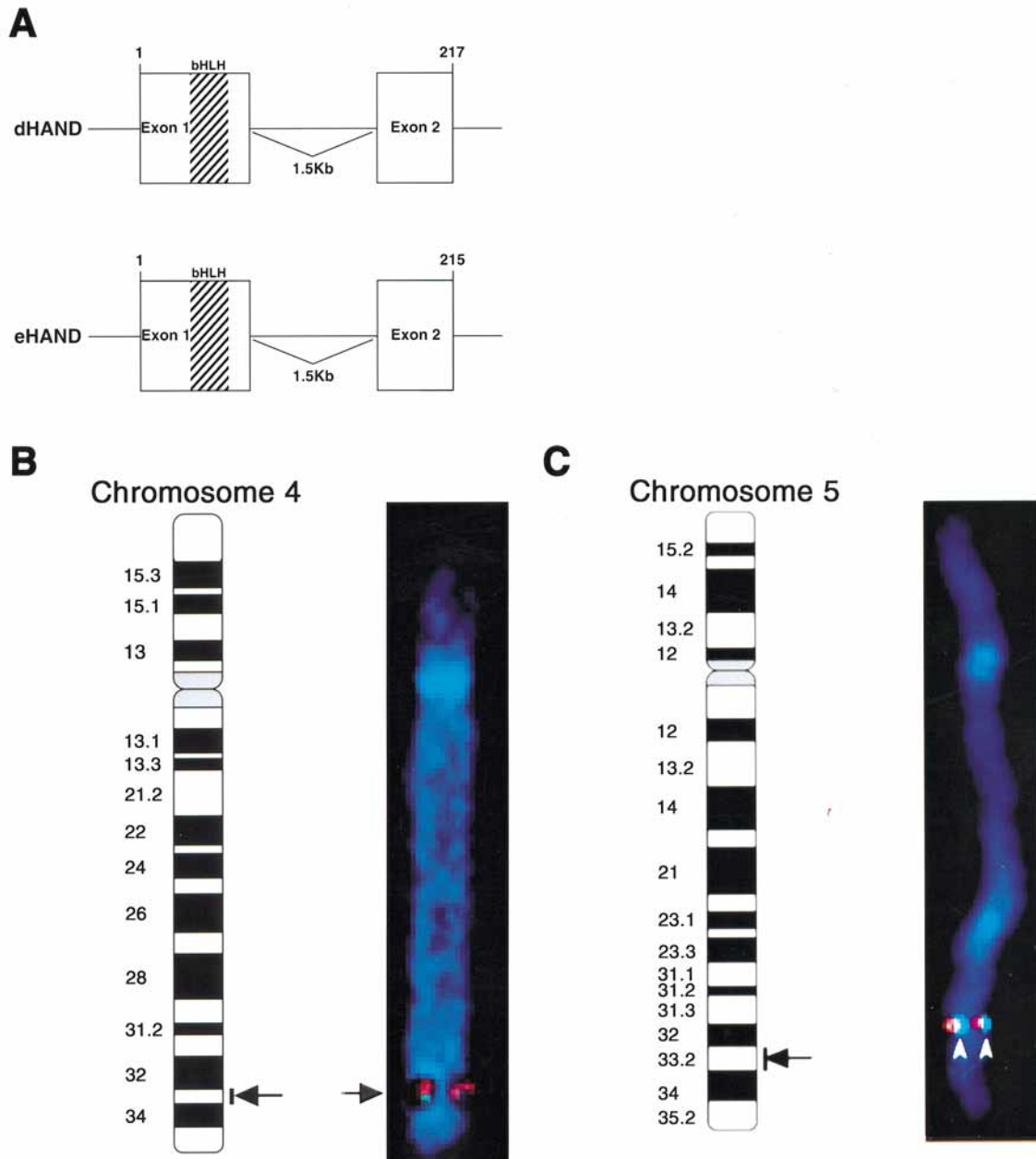


Figure 1 Genomic organization and FISH analysis of human *dHAND* and *eHAND*. Genomic organization of human *dHAND* and *eHAND* was similar with a single 1.5 kb intron located in identical positions in each, after the basic helix-loop-helix domain (bHLH), indicated in hatches (a). FISH analysis of human chromosome spreads in metaphase revealed that *dHAND* mapped to 4q33 (b) and *eHAND* mapped to 5q33 (c). Banding patterns are shown on left. Markers for 4q33 or 5q33 (pink) were used to confirm placement of *dHAND* or *eHAND* (green).

Expression of *dHAND* and *eHAND* in cardiomyopathies

Since *dHAND* and *eHAND* were expressed in the normal adult heart, we searched for alterations of expression in acquired heart disease. Biopsies of hearts from 15 patients with various forms of cardiomyopathy were obtained. Details of age, sex, diagnosis and cardiac function are shown in Table

1. The cardiomyopathies examined are generally associated with upregulation of fetal cardiac-specific genes. Semi-quantitative PCR with *dHAND* and *eHAND*-specific primers was performed. PCR products were quantified between 20 and 32 cycles of amplification to establish the linear range of amplification [Fig. 3(A)]. Based on these results, Southern blot was performed on samples after 26

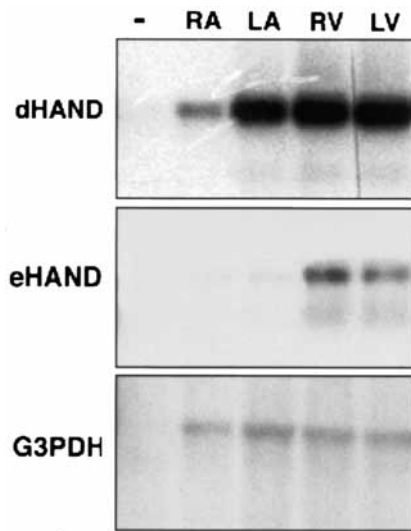


Figure 2 Chamber-specific analysis of human *HAND* gene expression. *dHAND* was expressed in both atria and both ventricles of the adult non-diseased heart as determined by semi-quantitative RT-PCR. Expression in the right atrium (RA) was diminished compared to the left atrium (LA), right ventricle (RV) and left ventricle (LV). *eHAND* expression was detected in the RV and LV, but was diminished in the RA and LA. Integrity and amount of RNA in each tissue was determined by G3PDH expression. PCR products were analysed in the linear range of amplification (26 cycles) and subjected to Southern blot. Negative control (–) is representative of non-reverse transcribed RNA samples of each tissue.

cycles of amplification and blots hybridized with ^{32}P -labelled *dHAND* or *eHAND* cDNA. We found that human *dHAND* expression was not significantly altered in the patients examined with hypertrophic, dilated, ischemic or sarcoid cardiomyopathies, compared to normal controls ($n=3$) in the same experiment [Fig. 3(B)]. Surprisingly, human *eHAND* expression was significantly downregulated in six of six patients with ischemic cardiomyopathy and six of six patients with idiopathic dilated cardiomyopathy [Fig. 3(B)]. In contrast, its expression was unchanged, compared to normal controls ($n=3$), in the few patients examined with hypertrophic or sarcoid cardiomyopathy, indicating that *eHAND* dysregulation was present in some but not all forms of cardiomyopathy. Amplification of *eHAND* beyond the linear range followed by Southern blot yielded similar results as shown with virtually undetectable levels of *eHAND* in hearts with dilated or ischemic cardiomyopathy.

Discussion

This study demonstrates that the *HAND* genes are expressed in the human adult heart in a chamber-specific fashion, with *eHAND* mostly restricted to the ventricular chambers. The downregulation of human *eHAND* in those forms of cardiomyopathy that involve ventricular thinning may provide a marker for idiopathic dilated and ischemic cardiomyopathy.

The high degree of conservation of the amino acid sequence of both *dHAND* and *eHAND* across species suggests functional conservation of these transcription factors, with *dHAND* being the more conserved of the two. Assignment of *dHAND* and *eHAND* to chromosomes 4q33 and 5q33, respectively, suggests an interesting hypothesis of gene duplication involving the *HAND* genes. Because chromosome 4q and 5q share over 13 pairs of paralogous genes along the length of the chromosome, it has been suggested that a tetraploidization resulted in the similarities between the two chromosomes and that part of 5q arose from 4q.²⁷ This observation, along with the remarkable conservation of genomic structure shown here, is consistent with a model in which *eHAND* arose from a duplication of the region surrounding *dHAND*.

A “4q deletion syndrome” has been described in a small group of patients and involves developmental, craniofacial, musculoskeletal and cardiac defects.^{25,28} Analyses of several series of patients have narrowed the critical region of 4q for this

Table 1 Clinical characteristics of patients with cardiomyopathy

No.	Gender	Age (years)	Diagnosis	NYHA class	Ejection fraction (%)
1	Female	17	HCM	4	70
2	Male	51	HCM	2	60–69
3	Male	40	DCM	3	15
4	Female	62	DCM	3	25–29
5	Female	32	DCM	4	<20
6	Female	36	DCM	4	20
7	Male	69	DCM	1	20
8	Male	71	DCM	4	13–19
9	Male	59	ICM	4	<25
10	Male	57	ICM	3–4	<20
11	Male	58	ICM	4	<20
12	Male	63	ICM	4	20
13	Male	58	ICM	4	25
14	Male	65	ICM	NA	20
15	Male	41	SCM	2	50–59

NYHA class, New York Heart Association functional class; HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; ICM, ischemic cardiomyopathy; SCM, sarcoid cardiomyopathy; NA, not available.

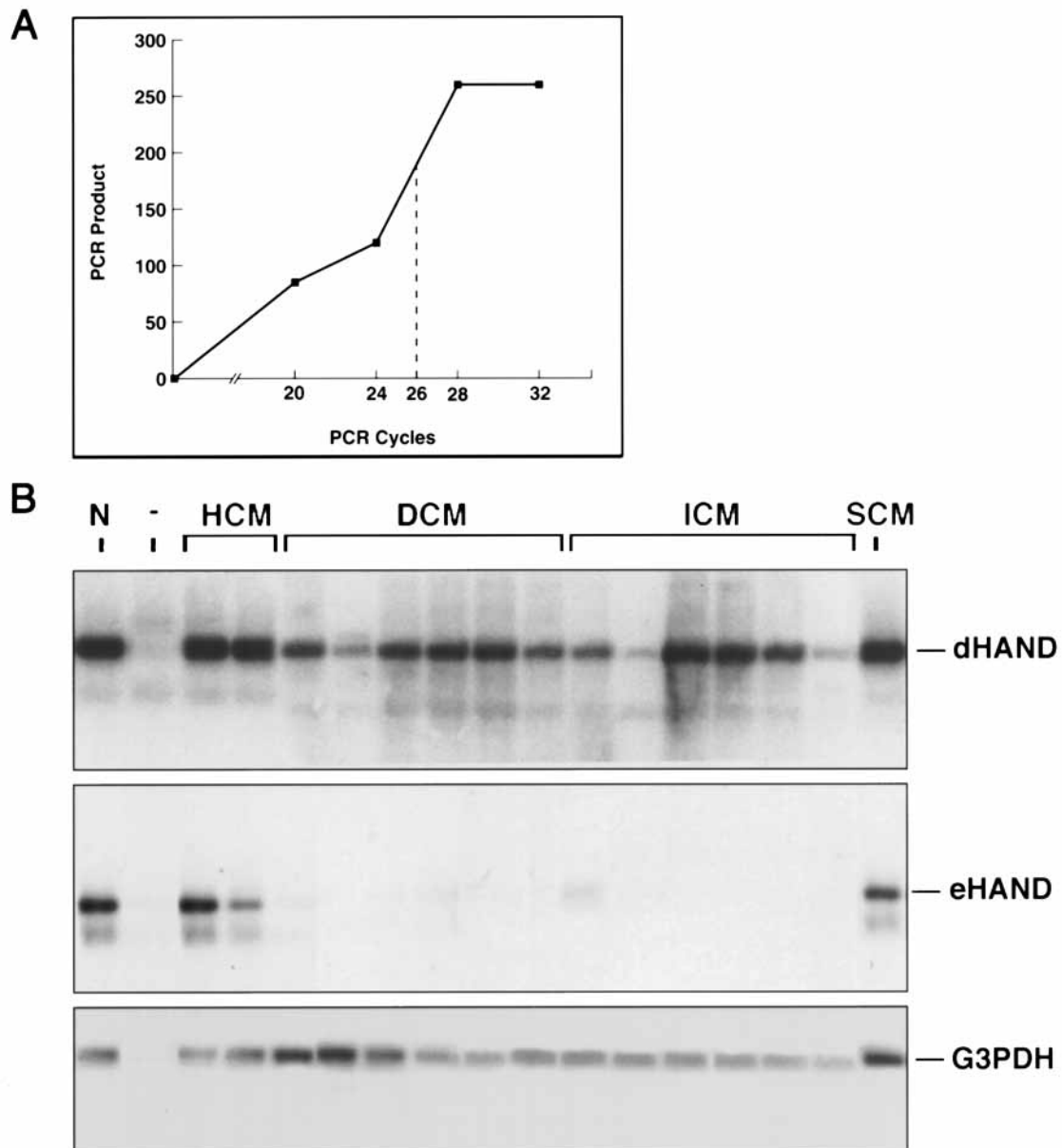


Figure 3 *dHAND* and *eHAND* expression in cardiomyopathies. *HAND* gene expression was assayed by semi-quantitative RT-PCR from hearts of 15 patients with a variety of cardiomyopathies and from three normal hearts. Representative quantification of RT-PCR products up to 32 cycles using ImageQuant software is shown in (a). Equal amounts of RNA for each sample resulted in similar curves demonstrating linear range of amplification. Data in (b) represents 26 cycles of amplification for each cDNA sample. *dHAND* expression was not significantly changed compared to normal (N) in all types of cardiomyopathies tested. *eHAND* transcripts were unaffected in two patients with hypertrophic cardiomyopathy (HCM) and one with sarcoid cardiomyopathy (SCM). However, *eHAND* transcripts were severely diminished or undetectable in 6/6 patients with dilated cardiomyopathy (DCM) and 6/6 patients with ischemic cardiomyopathy (ICM). RNA integrity and loading was monitored by G3PDH expression. PCR products were analysed in the linear range of amplification and subjected to Southern blot and hybridized with *dHAND* or *eHAND*-specific radiolabeled probes. Negative control (-) lane is representative of non-reverse transcribed RNA for each tissue sample. Expression of all normal hearts were equal and represented in lane (N).

syndrome. Terminal deletion of 4q distal to 4q31 resulted in cleft palate in 16/19 cases and cardiac defects in 11/19.²⁸ Eight reported cases of deletions

distal to 4q33 also had cardiac and craniofacial abnormalities.^{29,30} Of particular significance is a report of an interstitial deletion of 4q31.22-q34.2

that resulted in a phenotype resembling 4q31ter deletion.³¹ These data suggest that the genes contributing to 4q deletion syndrome reside between 4q31–4q33. Because *dHAND* maps to 4q33 and plays a role in craniofacial,¹⁵ cardiac¹¹ and limb development,^{32,33} it is tempting to speculate that deletion of *dHAND* contributes to the “4q31 deletion” syndrome. However, more definitive genetic analyses will be necessary to ascribe a role for *dHAND* in such defects. Mutation analysis of *dHAND* in patients with hypoplastic right ventricle conditions and aortic arch anomalies, as observed in *dHAND*-null mice, may reveal a specific role for *dHAND* in congenital heart disease.

The specific expression of *dHAND* and *eHAND* in the human adult heart indicates a possible function in heart maintenance. The ventricular expression of human *eHAND* suggests it may have functional properties related to ventricular rather than atrial function, similar to that observed for the *Irx4* gene.³⁴ More importantly, the downregulation of *eHAND* in dilated and ischemic forms of cardiomyopathy was striking. Although we cannot make any conclusions regarding *HAND* expression in hypertrophic or sarcoid cardiomyopathies, alterations in *eHAND* expression may serve as a molecular marker for certain types of cardiomyopathies. Numerous cellular pathways have been implicated in cardiac dysfunction including dysregulation of calcium-dependent pathways,⁵ stress-responsive MAP kinase cascades⁶ and β -adrenergic signaling.⁷ Activation of apoptotic pathways may also play a role in the evolution of dilated and ischemic cardiomyopathies, contributing to thinning of the ventricle and progressive cardiac dysfunction.³⁵ Alterations in the balance of survival pathways and apoptotic pathways following myocardial injury may also be involved in the evolution of cardiomyopathies. Given the requirement of *dHAND* in cell survival during development¹⁵ and the likely redundancies between *dHAND* and *eHAND*,⁹ it is tempting to speculate that the down-regulation of *eHAND* in advanced forms of cardiomyopathy may alter the delicate balance of cell survival and death in cardiomyocytes. Future studies will be necessary to determine the precise significance of *eHAND* downregulation in cardiomyopathy.

The studies presented here provide a framework to begin to study the potential roles of the bHLH proteins, *dHAND* and *eHAND*, in human congenital and acquired diseases. Utilizing a combination of mouse and human investigations, it will be important to develop an understanding of the precise function of the *HAND* proteins during development and maintenance of the heart.

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