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Remodeling of the Hypertrophied Human Myocardium by Cardiac bHLH Transcription Factors

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Abstract The basic helix-loop-helix transcription factors eHAND and dHAND are involved in developmental cardiac growth and differentiation. We investigated HAND gene expression in the normal and in the hypertrophied right and left ventricle of patients with tetralogy of Fallot (ToF) and hypertrophic obstructive cardiomyopathy (HOCM). HAND mRNA was constitutively expressed in the hypertrophied heart and increased in the hypertrophic tissue of both patient groups. HAND genes had a complementary left-right cardiac asymmetry of expression with dHAND predominantly in the right and eHAND in the left ventricle. The two cardiac bHLH factors have the ability to form heterodimers with the ubiquitous bHLH protein E12, subsequently recognizing E-boxes in the promoter region of target genes like ALC-1. We found a highly significant positive correlation between HAND and ALC-1 mRNA. The total ALC-1 protein level in ToF was smaller than in HOCM, although ALC-1 mRNA as well as HAND mRNA levels were significantly higher. ToF patients expressed around four times more ALC-1 mRNA for similar amounts of ALC-1 than HOCM patients. Suggesting disturbed ALC-1 translation in ToF, we found ALC-1 antisense mRNA expression in the hypertrophied, but not in the normal, ventricles. The higher the antisense/sense ALC-1 mRNA ratio, the lower ALC-1 protein was expressed. J. Cell. Biochem. 74:551–561, 1999.

Key words: tetralogy of Fallot; hypertrophic cardiomyopathy; ALC-1; antisense; eHAND; dHAND

Members of the basic helix-loop-helix (bHLH) transcription factors of the MyoD family are involved in the differentiation and maturation of skeletal muscle [Olson and Klein, 1994]. Recently, an additional bHLH familiy involved in the differentiation process of cardiomyocytes, was introduced, designated as eHAND and dHAND (also HAND1/Thing-1/HXT and HAND2/Thing-2/HED), having a large homology with the MyoD family [Srivastava et al., 1995]. The cardiovascular system is derived largely from lateral mesoderm and neural crest cells, in which eHAND and dHAND are expressed at high levels. In the mouse, their level of expression increases as the heart tube forms and starts to loop. Later during cardiac development, eHAND expression is restricted to the future left ventricle, while dHAND expression is restricted to the future right ventricle [Srivastava et al., 1997]. In the mouse, the development of the right ventricle failed upon dHAND gene targeting [Srivastava et al., 1997], while upon germline mutation of the eHAND gene, cardiac development failed to progress beyond the cardiac looping stage [Firulli et al., 1998]. Therefore, both HAND transcription factors are important for cardiac looping, ventricular specification, and growth [Thomas et al., 1998]. According to its significant role we investigated HAND gene expression in the normal and hypertrophied human ventricle. We found increased HAND mRNA expression in both the hypertrophied left and right ventricle of patients with hypertrophic obstructive cardiomyopathy (HOCM) and tetralogy of Fallot (ToF), respectively. The HAND transcription factors can heterodimerize with the ubiquitous bHLH protein E12, subsequently interacting with

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E-boxes [Cserjesi et al., 1995; Hollenberg et al., 1995]. An E-box which interacts with muscle-specific regulatory proteins of the basicHLH family exists in the ALC-1 (atrial myosin light chain-1) promotor region [Catala et al., 1995], suggesting the ALC-1 gene as a target for transcription regulation by HAND factors. Weak binding of the HAND transcription factors to E-boxes has been described [Cserjesi et al., 1995].

In addition to HAND/ALC-1 correlation, we analyzed the ratio between ALC-1 mRNA and protein expression in the human ventricle. These studies were performed because of the important function of ALC-1 in regulating human heart contractility. We were recently able to demonstrate that both cross-bridge cycling kinetics and contractility of human heart fibers increased upon expression of ALC-1 [Morano et al., 1996, 1997]. These results were finally verified in an ALC-1 transgenic mouse model [Fewell et al., 1998]. We observed a close positive correlation between ALC-1 mRNA and protein in HOCM, but not in ToF. In fact, compared with HOCM, ToF patients expressed about four times more ALC-1 mRNA for similar amounts of ALC-1. To analyze the cause of this observation, we investigated the existence and expression level of naturally occurring antisense ALC-1 mRNA in the human ventricles.

Expression of naturally occurring antisense mRNA for sarcomeric protein genes has recently been demonstrated for the rat heart myosin heavy chain genes [Luther et al., 1997, 1998] as well as for the skeletal muscle fast-twitch myosin heavy chain gene [McCarthy et al.,1983]. Because of the formation of duplex mRNA, naturally occurring antisense mRNA has the capacity to quench mRNA translation and hence may play a role in the regulation of cardiac contractility. For the first time, we demonstrate herein the expression of naturally occurring antisense ALC-1 mRNA in the human ventricle. ALC-1 antisense mRNA expression in patients with ToF was statistically higher compared with control or HOCM hearts. We suggest that ToF patients required higher ALC-1 mRNA for ALC-1 translation due to the presence of high levels of endogenous antisense ALC-1 mRNA.

MATERIALS AND METHODS

Patient Population

Right ventricular infundibular tissue was obtained from 8 unrelated patients undergoing repair of congenital heart disease in the German Heart Center (Berlin, Germany). All patients suffered from ToF, resulting in cyanosis and dyspnea. Surgical treatment consisted of the repair of the malformations [Kirklin et al., 1992]. Tissue from the hypertrophied interventricular septum was obtained from 18 unrelated patients with HOCM undergoing open heart surgery at the department for thoracic and cardiac surgery at the Heinrich Heine University (Düsseldorf, Germany). All patients had severe HOCM predominantly of the interventricular septum with severe obstruction of the left ventricular outflow tract and suffered from dyspnea, angina, and/or syncopes. Surgical treatment consisted in all cases of transaortal subvalvular myectomy [Morrow et al., 1975; Schulte et al. 1993]. The clinical evaluation consisted of physical examination, electrocardiography (ECG), and echocardiography. Interventricular tissue of the left ventricle was taken from six normal hearts that could not be transplanted due to technical reasons. Informed consent was obtained from all patients and our protocol was approved from the local ethic committees. All tissue samples were immediately frozen in liquid nitrogen after resection. All biochemical experiments on the protein level were performed with tissue samples immediately frozen in liquid nitrogen after resection and stored at −80°C. Original pictures in this paper are representative examples for each group. They were not taken from the same patient in each case.

ALC-1 Analysis

Myosin light chains were analyzed by a high-resolution two-dimensional polyacrylamide gel electrophoresis (2D PAGE) technique as described earlier [Morano et al., 1996]. In brief, isoelectric focusing (first dimension) was performed in glass capillaries (12.5 cm long, 1-mm inner diameter) using the pH gradient 4.5–5.5 (Pharmalytes; Pharmacia, Sweden). The gels were run overnight at 600 V constant load for the first dimensional separation. The second dimension was sodium dodecyl sulfate (SDS)-electrophoresis, using slab gels 10.5 × 9.5 cm, 1 mm thick. The gels were stained in Coomassie blue, and the MLC were evaluated by computer-assisted scanner densitometry (ScanPack, Biometra, Germany). Densitometric analysis of the ALC spots was always within the linear portion of the relation between protein concentration

and optical density (Lambert-Beer Law). Expression of ALC-1 was determined 4–6 times for each patient. ALC-1 protein expression is given as percentage of total MLC-1 (i.e., ALC-1 + VLC-1 = 100%). The results were highly reproducible.

RNA Preparation

Total RNA was prepared according to the protocol of [Chomczynski and Sacchi, 1987]. In brief, small samples of minced tissue was dissolved in 800 µl of 4 mol/L guanidium thiocyanate, 25 mmol/L sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 mol/L 2-mercaptoethanol and homogenized. A total of 80 µl of 2 mol/L sodium acetate, pH 4, 800-µl water-saturated phenol (Roth), and 160 µl chloroform/isoamyl alcohol (25:1) were added. After centrifugation at 12,000*g* for 20 min at 4°C, the aqueous phase was transferred into a fresh tube, 800 µl isopropanol was added, and the sample was centrifuged at 5,000*g* for 10 min. The pellet was washed with 75% ethanol and the RNA dissolved in 20 µl water. The concentration was determined by ultraviolet (UV) absorption. The ratio of optical density at 260 nm and 280 nm was 1.8–2.0 in all cases. The integrity of the RNA was determined by examining the 28S and 18S rRNA bands in ethidium-bromide-stained agarose gels using a 23S/16S rRNA from *Escherichia coli* (Boehringer, Mannheim, Germany) as standards.

Quantitative Reverse Transcription Polymerase Chain Reaction

For reverse transcription of mRNA into cDNA 1 µg of total RNA was used. RNA was denaturated for 5 min at 70°C and then reverse-transcribed by incubation for 60 min at 42°C in the presence of 0.5 mM dNTP, 10 µM dithiothreitol, 25 pmol/L OT primer (Gibco-BRL), 1 U MMLV superscript reverse transcriptase (Gibco-BRL), pH 8.3, 75 mmol/L KCl , and 3 mmol/L $MgCl_2$. The reaction was stopped by heating at 70°C for 10 min.

cDNA was amplified using 1.5 U TAQ-DNA polymerase (Gibco-BRL) in an assay mixture containing 1 µmol/L of the respective oligonucleotide primers, 10 mmol/L Tris-HCl, pH 8.3, 1.5 mmol/L $MgCl_2$, and 20 µmol/L dNTP. The mixture with a final volume of 50 µl was overlaid with mineral oil and amplified in a thermal cycler (Biometra) for 22 cycles for ALC-1 sense and antisense and β-actin and 34 cycles for eHAND and dHAND, respectively. Denaturation was carried out at 94°C for 1 min, followed by an annealing step 58°C for 1 min and an extension step at 72°C for 1.5 min. Products were analyzed by 8% polyacrylamide gel electrophoresis (PAGE), stained with ethidium bromide, photographed, and evaluated by film densitometry (ScanPack, Biometra, Germany). The relative amount of cDNA was expressed as a percentage of the total sum of peak areas. Quantification of PCR products was performed within the linear range of cycle number versus the logarithm of accumulated PCR products as already described [Luther et al., 1997].

For the detection of ALC-1 antisense mRNA, reverse transcription was performed with an antisense specific oligonucleotide [Marino et al., 1991]. The sequence of this forward primer was ATGGCTCCCAAGAAGCCTGAG; the position was 1–21 according to the published sequence [Zimmermann et al., 1990]. For ALC-1 sense-mRNA detection OT primers were used in the reverse transcription. For all other primers, see Table I.

TABLE I. Oligonucleotides Used in This Study

	Forward	Reverse	
ALC-1 Coding region	CCGACTGGAGAGATGAAGATC (position 199–219)	GAACGTCTCAAAGTCCAG-CATC (position 348–327)	cDNA fragment of 150 bp
ALC-1 Reverse transcription for antisense RNA	ATGGCTCCCAAGAAGCCTGAG (position 1–21)		
eHAND	ACCAGCTACATCGCCTACCT-GATG (position 417–441)	TCCCTTTTCCGCTTGCTTTCA (position 538–516)	cDNA fragment of 122 bp
dHAND	CGACCAGAACGGAGAGGCG (position 474–492)	GTCGTTGCTGCTCACTGTGC (position 590–572)	cDNA fragment of 117 bp
β-Actin	GAGACCTTCAACACCCCAGCC (position 414–435)	TCAGGGCAGCGGAACCGCTCA (position 817–797)	cDNA fragment of 404 bp

For the PCR, cDNA from the reverse transcription was amplified using specific oligonucleotides for both sense and antisense ALC-1 mRNA (Table I). This led to a predicted cDNA fragment of 150 bp [Zimmermann et al., 1990]. The product was coamplified with oligonucleotides for human β-actin. The resulting cDNA consisted of 404 bp.

To examine the expression pattern of eHAND and dHAND in human adult ventricular myocardium, we amplified cDNA of normal hearts and of myocardial tissue of ToF patients. For each patient, PCR for eHAND, dHAND, and for β-actin was performed in separate tubes. β-Actin was used as internal standard. Control experiments were carried out to ensure that the results are in the exponential phase of product amplification and amplified with equal efficiency. The sequences for the oligonucleotides for amplification of eHAND and dHAND were published previously [Russell et al., 1997] or taken from Genbank (accession U 40039)(Table I). eHAND amplification caused a cDNA with 122 bp, the dHAND cDNA was a fragment of 117 bp. Analysis of the PCR products revealed the expected sequence (data not shown) and size. All primers, their position, the orientation and the fragment sizes are shown in Table I.

Separate experiments were performed to exclude amplification of contaminating genomic DNA. Therefore, RNA was digested before reverse transcription by incubation with 40 U T2 RNAse (Gibco-BRL) at 37°C at in a buffer containing 50 mM sodium acetate (pH 4.5) and 2 mM EDTA for 30 min. For RT-PCR, probes were neutralized with 1 M NaOH to pH 7.2. In addition, equimolar amounts of prepared RNA were used instead of cDNA in the PCR cycling procedure. Up to 30 cycles, products were not obtained by this arrangement (not shown).

DNA Sequencing

All PCR products were analyzed commercially (Invitek, Berlin, Germany) by an ABI 373 DNA sequencer (Perkin-Elmer) using the ABI PRISM dye terminator cycle sequencing method and compared with the expected sequences. A total of 10–20 ng of agarose gel-purified PCR fragment and 3.2 pmol/L primer were used in a total volume of 20 µl. The sequenzing reactions were performed on an Gene Amp PCR system 2400 (Perkin-Elmer) and run on a 5.5% gel on 32 W for 11 h.

Statistical Analysis

All statistical analysis were performed using commercially available statistic programs (Enzfitter, Epistat, Prism) on Apple Macintosh or on an IBM-compatible PC. Values are means ±SEM. Significance analysis was performed using the Student's t-test for unpaired values. Regression analysis was performed by the least-squares method.

RESULTS

Expression of eHAND and dHAND mRNA

We compared HAND gene expression on mRNA level in right ventricular tissue of 8 Fallot patients and in left ventricular tissue of 18 HOCM patients and of 6 normal hearts. A signal on ethidium bromide stained agarose gels was obtained after 34 cycles. To examine the expression pattern of eHAND and dHAND in human adult ventricular myocardium, we amplified cDNA with oligonucleotides specific for either eHAND or dHAND. For each patient, PCR for eHAND, dHAND and for β-actin was performed in separate tubes. β-Actin was used as internal standard. The results for total HAND gene expression were calculated as ratio of (eHAND + dHAND)/β-actin and for the specific HAND gene expression as ratio of eHAND/β-actin or dHAND/β-actin, respectively. The PCR product accumulation for eHAND and dHAND were in the exponential phase up to 34 cyles, for β-actin up to 22 cycles. Analysis of the PCR products revealed the expected sequence (data not shown) and size (Table I) as predicted from the published data (Fig.1).

In normal left ventricular human adult heart of eHAND and dHAND, expression could be detected. The eHAND/β-actin and dHAND/β-actin ratios were 0.31 ± 0.02 and 0.24 ± 0.1, respectively (Fig. 2). No right ventricular tissue of normal human heart was available. In right ventricular human adult myocardium of patients with ToF, dHAND expression was higher than eHAND expression. The dHAND/β-actin ratio was 2 ± 0.8, the eHAND/β-actin ratio was 0.8 ± 0.2 ($P < 0.01$), respectively. Both HAND genes were significantly higher ($P < 0.01$) than in normal heart.

In left ventricular myocardium of HOCM patients, eHAND was on a higher level than dHAND. The ratio for dHAND/β-actin was 0.75 ± 0.1 compared with an eHAND/β-actin ratio of 1.1 ± 0.25 ($P < 0.01$) (Fig. 2).

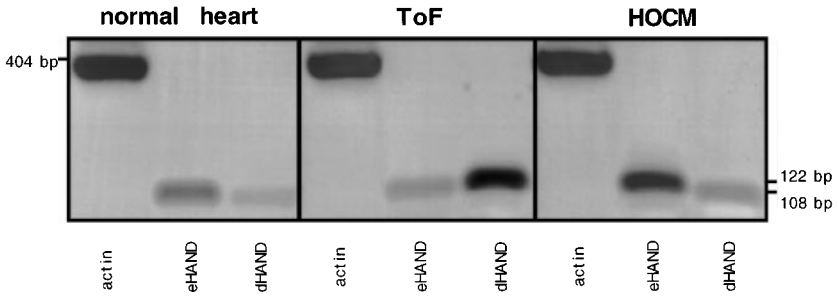

Fig. 1. Original photograph of reverse transcription-polymerase chain reaction (RT-PCR) of eHAND and dHAND. We investigated normal heart (left), right ventricular tissue of a tetralogy of Fallot (ToF) patient (center), and left ventricular tissue of a patient with hypertrophic obstructive cardiomyopathy (HOCM) (right). In each specimen, eHAND and dHAND mRNA were expressed but in the right ventricle of ToF patients dHAND mRNA was on a higher level than eHAND, while in the left ventricle of HOCM eHAND is expressed on a higher level than dHAND.

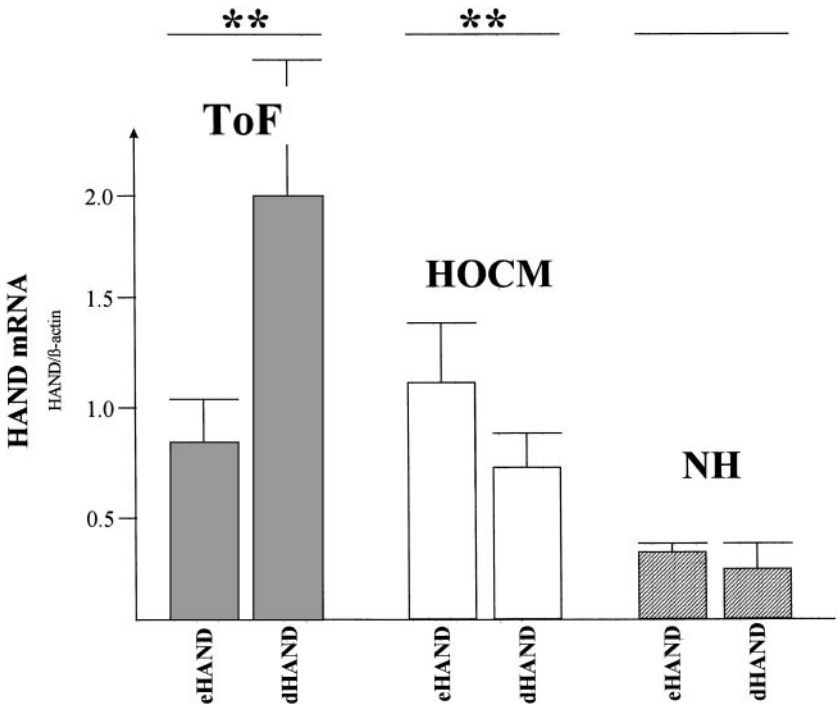

Fig. 2. Expression of eHAND and dHAND in the right ventricle of ToF patients, in the left ventricle of patients with hypertrophic obstructive cardiomyopathy (HOCM), and in the left ventricle of normal hearts. HAND mRNA expression is given as total of eHAND or dHAND, respectively, normalized to β-actin. Total amounts of HAND mRNA are significantly higher in HOCM and in ToF compared with normal heart ($P < 0.01$).

The difference between eHAND and dHAND expression in ToF and in HOCM was statistically significant ($P < 0.01$). Total HAND expression in ToF was significantly higher ($P < 0.01$) than in HOCM.

Expression of ALC-1 mRNA

We examined the expression of ALC-1 on the mRNA level in the tissue of patients with ToF, HOCM, and of normal hearts by RT-PCR. The strategy used was to amplify cDNA with oligonucleotides matching the protein coding region. In each case ALC-1 cDNA was coamplified with cDNA of β-actin as internal standard. The results were calculated as ratios of ALC-1/β-actin mRNA. Several control experiments were carried out to ensure that the measurements were made in the exponential phase of product amplification. ALC-1 and β-actin mRNA were amplified with equal efficiency (data not shown). The PCR signal obtained upon 22 cycles was well within the exponential phase of product amplification. As predicted from the cDNA sequence [Zimmermann et al., 1990], the PCR products from the ALC-1 coding region had 150 bp, from β-actin 404 bp (Fig.3). Furthermore, the PCR product revealed the expected sequence [Zimmermann et al., 1990] (data not shown).

In the normal human left ventricle, ALC-1/β-actin ratio was 0.27 ± 0.1.

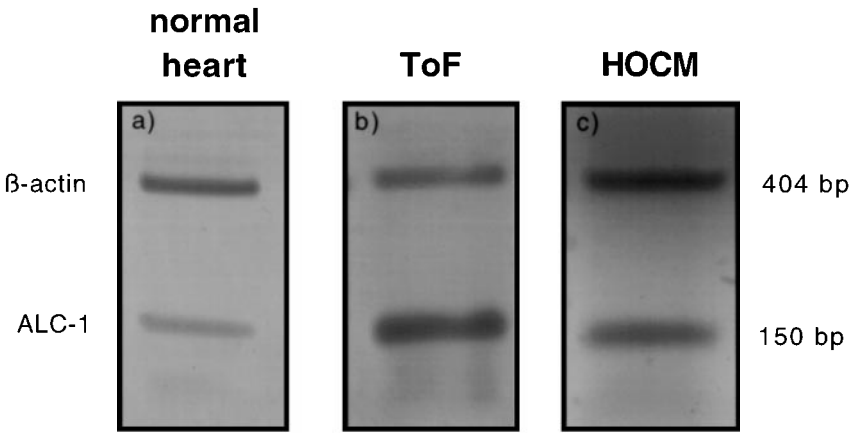

Fig. 3. Original photographs of reverse transcription-polymerase chain reaction (RT-PCR) of ALC-1 mRNA. We investigated tissue from normal heart (left ventricle) **(a)**, tissue from tetraology of Fallot (ToF) patients (right ventricle **(b)**, and tissue of patients with hypertrophic obstructive cardiomyopathy (HOCM) (left ventricle **(c)**. Coamplification with β-actin (top lane) was performed in all gels. Only low amounts of ALC-1 mRNA were detectable in normal hearts; however, there were high amounts in ToF and low amounts of ALC-1 mRNA in HOCM.

In the hypertrophied right ventricular tissue of Fallot patients, the mean ALC-1/β-actin mRNA ratio was 1.8 ± 0.3, ranging from 1.27 to 1.97, which was significantly higher than in normal left ventricle ($P < 0.01$).

In HOCM patients, ALC-1/β-actin mRNA ratio was 0.87 ± 0.4, which was significantly lower ($P < 0.01$) compared with ToF, but significantly higher than normal hearts ($P < 0.01$). The ALC-1/β-actin ratio of HOCM patients ranged from 0.38 to 1.8.

Correlation Between HAND mRNA and ALC-1 mRNA Expression

There was a significant positive correlation between the expression of HAND mRNA (i.e., the sum of eHAND and dHAND) and the level of ALC-1 mRNA. In normal hearts, HAND/β-actin ratio was 0.55. HAND/β-actin ratio of ToF patients ranged from 1.2 to 5.8 (mean 2.8). In left ventricular tissue of HOCM patients, HAND mRNA expression was 0.8–3.2 (mean 1.85) ($P < 0.05$). High levels of HAND mRNA correlated with increased ALC-1 mRNA expression (Fig. 4).

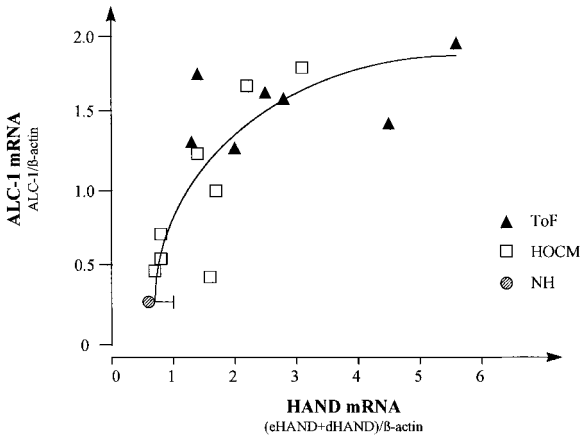

Fig. 4. Correlation between the expression of HAND and of ALC-1 mRNA. HAND mRNA is given as the sum of eHAND and dHAND, normalized to β-actin. ALC-1 mRNA levels are expressed as ratio of ALC-1/β-actin mRNA. High levels of HAND mRNA are related to high amounts of ALC-1 mRNA.

ALC-1 Protein Analysis

The myosin light chain (MLC) isoforms were analyzed according to their isoelectric point and molecular weight by 2D-PAGE. The atrial isoform of the essential light chain (ALC-1)displayed a higher molecular weight and was more acidic than the ventricular isoform (VLC-1). Figure 1 shows representative original gels of frozen tissue of the interventricular septum from patients with ToF and HOCM. Both tissues contained VLC-1 and the two ventricular isoforms of the regulatory light chains, VLC-2 and VLC-2*.

The ratio between essential light chains (MLC-1) and regulatory light chains (MLC-2) showed no significant difference between normal left ventricles (1.11 ± 0.3), right ventricles of ToF patients (1.26 ± 0.78) and HOCM left ventricular tissue (1.21±0.48). The atrial isoform of the regulatory light chain (ALC-2) was never observed in our tissue. Evaluation of the two ventricular MLC-2 isoforms (VLC-2 and VLC-2*) revealed a significant different ratio of and 4.4 ± 1.2 ($P < 0.01$) in HOCM patients compared *with* the VLC-2/VLC-2* ratio of 3.1 ± 0.3 in normal hearts. In the hypertrophied tissue of Fallot patients, this ratio was 3.9 ± 0.9, i.e., not significantly different from normal hearts, but significantly lower ($P < 0.01$) compared with HOCM.

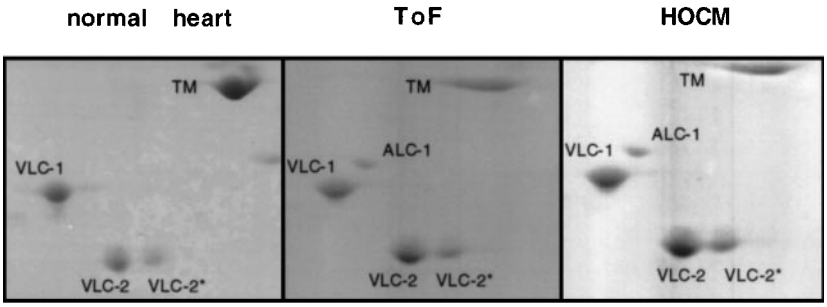

Fig. 5. Original photographs of 2D polyacrylamide gel electrophoresis (PAGE) analysis of myosin light chainn (MLC) of normal heart, tetralogy of Fallot (ToF), and hypertrophic obstructive cardiomyopathy (HOCM). All fibers contained VLC-1, VLC-2, and VLC-2*, but patients with ToF and HOCM contained ALC-1 as well. TM, tropomyosin; ALC-1, atrial essential light chain; VLC-1, ventricular essential light chain; VLC-2 and VLC-2, isoforms of the ventricular regulatory light chain.

In normal ventricles, ALC-1 could not be detected in the left ventricles; even heavily overloaded gels showed no traces of ALC-1 protein. In the right ventricular tissue of ToF patients (Fig. 5), ALC-1 expression was 3.9 ± 0.7, ranging between 0% and 5.1% ALC-1 (given in percentage of total MLC-1 , i.e., ALC-1 + VLC-1 = 100% = MLC-1).

In the left ventricles of HOCM patients (Fig. 5), we found considerable amounts of ALC-1, ranging from 0% to 32.5% ALC-1. The mean ALC-1 expression of patients with HOCM was 12.7 ± 1.7.

Correlation Between ALC-1 mRNA and Protein Expression

In HOCM patients, there was a significant positive correlation ($P < 0.01$) between the ALC-1 protein content and the ALC-1 mRNA content, in that the higher the ALC-1 mRNA the more protein was expressed (Fig. 6). Lowest levels of ALC-1 mRNA were found in normal hearts (0.27 ± 0.1) and some patients with HOCM and ToF. This coincided with the absence of ALC-1 protein. A threshold level for ALC-1 protein expression could be observed at 0.44 ALC-1/β-actin mRNA ratio.

In the hypertrophic ToF patients, there was no significant correlation between the ALC-1 protein and ALC-1 mRNA (Fig. 3). Although mRNA expression was on a high level (1.8 ± 0.3 ALC-1/β-actin ratio), ALC-1 protein expression was only 3.9 ± 0.7. For the same amount of protein, HOCM expressed an ALC-1/β-actin mRNA ratio of 0.47; i.e., ToF required about four times more ALC-1 mRNA for equal amounts of protein than HOCM.

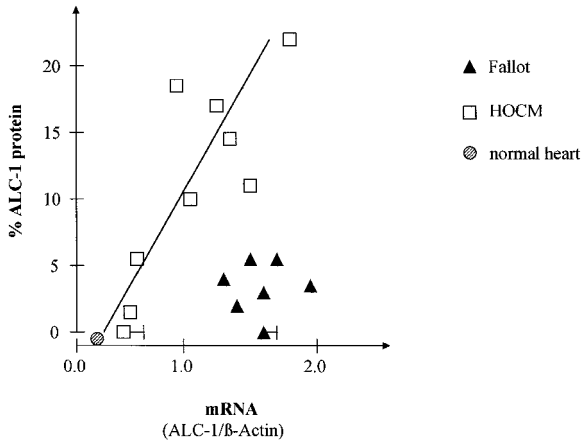

Fig. 6. Expression of ALC-1 in the hypertrophied hearts of patients with tetralogy of Fallot (ToF) and hypertrophic obstructive cardiomyopathy (HOCM). The diagram shows the correlation of ALC-1 protein and ALC-1 mRNA. mRNA content is given as ratio ALC-1/β-actin. ALC-1 protein expression is given as percentage of total MLC-1 (i.e., ALC-1 + VLC-1 = 100%). Note that protein levels in ToF remain low, despite a pronounced expression of ALC-1 mRNA.

Expression of Antisense ALC-1 mRNA

To quantify antisense ALC-1 mRNA, 22 cycles were enough to obtain an ethidium bromide signal of antisense ALC-1 mRNA in normal heart and in both patient groups (Fig. 7). cDNA amplified upon antisense-mRNA reverse transcription revealed the predicted size, i.e., 150 bp for the antisense cDNA.

In the left ventricular tissue of normal human heart, no ALC-1 antisense mRNA could be detected. We cannot exclude, however, that upon increasing the number of PCR cycles, a slight ALC-1 antisense mRNA signal might be obtained.

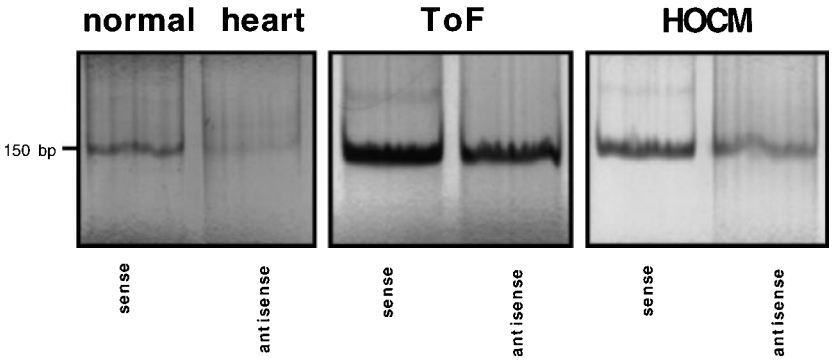

Fig. 7. Original photographs of acrylamide gels with ALC-1 sense and antisense mRNA. No antisense mRNA of ALC-1 is found in normal heart (left), while antisense mRNA in tetralogy of fallot (ToF) and hypertrophic cardiomyopathy (HOCM) was clearly visible after 22 polymerase chain reaction (PCR) cycles. The average antisense level in ToF was about twofold higher than in HOCM.

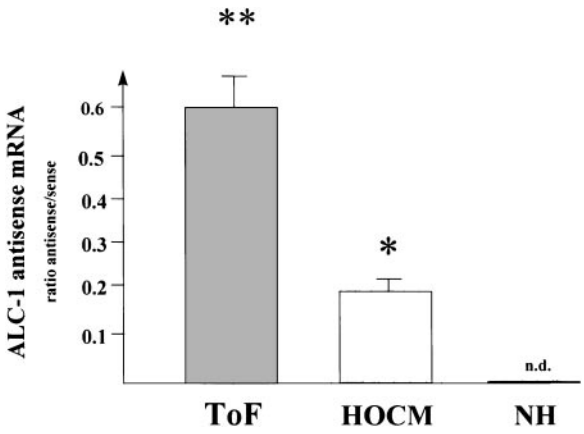

Fig. 8. Comparison of ALC-1 antisense levels in tetralogy of Fallot (ToF), hypertrophied obstructive cardiomyopathy (HOCM), and normal heart. Antisense expression was given as ratio of antisense/sense mRNA of ALC-1. Compared with normal heart antisense levels were significantly higher in HOCM ($P < 0.05$) and ToF ($P < 0.01$). n.d., not detectable.

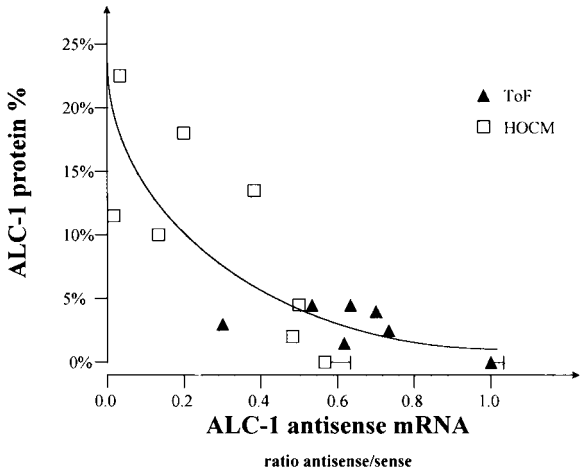

Fig. 9. Diagrammatic representation of the correlation between the level of ALC-1 antisense mRNA and ALC-1 protein expression. Antisense expression was given as ratio of antisense/sense mRNA of ALC-1. ALC-1 protein expression is given as percentage of total myosin light chain-1 (MLC-1) (i.e., ALC-1 + VLC-1 = 100%). High levels of antisense obviously suppress the ALC-1 protein expression. This is found predominantly in the right ventricle of tetralogy of Fallot (ToF) patients.

Densitometric analysis and quantification of the RT-PCR signals revealed that in ToF, the mean ratio of ALC-1 antisense/sense mRNA was 0.62 ± 0.08 ranging from 0.3 to 1.0. In HOCM, the antisense/sense mRNA ratio was significantly lower (0.18 ± 0.01) ($P < 0.01$), ranging from 0 (no antisense but sense mRNA) to 0.57 (Fig. 8). There was an exponential relationship between ALC-1 antisense/sense ratio and ALC-1 protein, i.e., high amounts of antisense correlated with smaller amounts of protein as demonstrated in Figure 9.

DISCUSSION

Cardiac hypertrophy is an adaptive response to improve contractility and to normalize wall stress and to compensate for increased work load. It is accompanied by qualitative and quantitative changes of gene expression [Schaub et al., 1998]. Analysis of nuclear transcription factors involved in the cardiac hypertrophy process is, therefore of special interest. In the mouse, two bHLH transcription factors of the heart have recently been described, designated as eHAND and dHAND. Their expression level increased as the heart tube forms and starts to loop. In knockout and antisense experiments, HAND transcription factors revealed to be important for left and right ventricular development [Srivastava et al., 1997; Firulli et al., 1998; Thomas et al., 1998]. According to the significant role of HAND transcription factors in cardiac differentiation and growth, they may also be important in the cardiac hypertrophy process. We found constitutive HAND gene expression at low levels in the normal human left ventricle, an observation in accordance with Russell et al. [1998]. Compared with the normal human left ventricle, we demonstrated for

the first time a highly significant upregulation of HAND mRNA in the hypertrophied right (fourfold) and left ventricle (2.5-fold) of patients with ToF and HOCM, respectively. Normal right ventricular tissue was not available for our studies. dHAND mRNA levels were predominant in the right ventricular tissue of ToF, while eHAND mRNA predominated in the left ventricular tissue of HOCM. In fact, during cardiac development of the mouse, eHAND was restricted to the left-sided sytemic ventricle while dHAND was restricted to the right-sided pulmonary ventricle [Srivastava et al., 1997], an expression pattern that appears to be maintained in the hypertrophied human adult heart.

Dimerization of bHLH proteins of the MyoD-family with E12 transcription factors results in the formation of a DNA-binding domain that recognizes the E-box consensus sequence (CANNTG) in the promoter region of target genes. Similarly, the two cardiac bHLH factors, eHAND and dHAND, have the ability to form heterodimers with the ubiquitous bHLH protein E12, and to interact with E-box elements of target genes [Cserjesi et al., 1995]. An E-box, specific for muscle regulatory proteins of the MyoD bHLH family, exists within the first 530 bp of the promotor region of the human atrial myosin light chain gene (ALC-1) [Catala et al., 1995]. Since E-boxes have been shown to be sufficient for ALC-1 transcription regulation we analyzed the expression of the ALC-1 gene as a downstream target for HAND transcription factors. Indeed, we found a highly significant positive correlation between HAND and ALC-1 mRNA expression in the hypertrophied human ventricles in that the higher the HAND mRNA the higher was the ALC-1 mRNA expression.

In addition to the HAND proteins, several more factors may be involved in ALC-1 upregulation in the hypertrophied heart. Besides the E-box, we identified a consensus binding sequence for the zinc-finger transcription factor GATA 4 (TGATAG) at −889 of the human ALC-1 gene promotor. GATA mediates the hypertrophic response in the heart [Herzig et al., 1997]. Recently, the molecular events that could explain increased GATA 4 activity in the hypertrophied heart were presented [Molkentin et al., 1998]; upon increased intracellular Ca^{2+} concentration, NF-AT3, a member of the "nuclear factors of activated T cells" (NF-AT), becomes dephosphorylated by the Ca^{2+}-dependent protein phosphatase calcineurin, translocated to the nucleus, and interacted with GATA 4, increasing its DNA binding activity. In addition NF-AT3 may have the capability to directly activate ALC-1 transcription due to the presence of the NF-AT3 consensus sequence (GGAAAAT) in the human ALC-1 promotor (−556 to −563). From our own work we conclude, that NF-AT3 and NF-AT3/GATA 4 activity alone could not be sufficient for ALC-1 translation: a large proportion of ToF and HOCM patients exhibited low ALC-1 mRNA levels that were close to the normal human ventricle despite pronounced cardiac hypertrophy. These patients showed HAND mRNA levels similar to the values found in the normal human heart. We therefore suggest that ALC-1 gene transcription is regulated by several transcriptions in a coordinated manner. Insufficient expression of one of them, e.g., the HAND transcription factors, did not permit expression of ALC-1 mRNA.

As mentioned above, the normal human heart expressed virtually no ALC-1 on the protein level. In these ventricles, ALC-1 mRNA as well as HAND mRNA expression remained low. We conclude that in the normal human ALC-1, mRNA is below a threshold level required for ALC-1 translation. It should be noted that in some patients with ToF and HOCM, no ALC-1 protein could be detected. The same patients revealed HAND and ALC-1 mRNA as low as in normal ventricles, i.e., below the critical threshold level. In our experiments, ALC-1 protein could not be detected up to a ALC-1/β-actin mRNA ratio of 0.44. Above that value, there was a linear relationship between ALC-1 mRNA and ALC-1 protein in HOCM patients.

In addition to the threshold phenomenon involved in the regulation of ALC-1 translation, another interesting finding in this work is that ToF patients required about four times more ALC-1 mRNA than HOCM for expression of the same amount of ALC-1 protein. Thus, in ToF patients, translation efficiency may be disturbed. One initial speculation to explain this observation was that naturally occurring antisense mRNA generated from the ALC-1 gene exist in the ToF ventricles. This seems to be rational, as antisense mRNA has the ability to form duplex mRNA, thus quenching translation efficiency [McCarthy et al., 1983; Boheler et al., 1992]. Furthermore, endogenous antisense mRNA raised from genes coding for sarcomeric proteins have already been described in the heart [Luther et al., 1998]. We observed for

the first time endogenous antisense ALC-1 mRNA expressed in the hypertrophied ventricles of ToF and HOCM. It remained undetectable by our method in the normal left ventricle. We found a clear dependency of ALC-1 protein from the ratio antisense/sense ALC-1 mRNA in that the higher the ratio the lower was the ALC-1 translation product. ToF patients in general exhibited a higher antisense/sense ratio than that of HOCM patients. A ToF patient with an antisense/sense ALC-1 mRNA ratio of 1.0 revealed virtually no ALC-1 protein; i.e., the ALC-1 message was completely quenched upon expression of equimolar amounts of antisense and sense ALC-1 mRNA. The uncommonly high ALC-1 mRNA expression in ToF may therefore be considered an adaptation to overcome the presence of high endogenous antisense ALC-1 mRNA and to keep the sense ALC-1 mRNA at an upper threshold level.

Once the upper threshold level is reached, expression of the ALC-1 is permitted in the human ventricle. In fact, we could demonstrate recently, that ALC-1 expression increased the actin interaction kinetics of the human molecular motor, thus improving cardiac contractility [Morano et al., 1996, 1997]. We suggest that the upregulation is a secondary response to maintain cardiac function in the face of primary lessions. This could be finally verified in an animal model, overexpressing ALC-1 [Fewell et al., 1998].

In summary, HAND mRNA and ALC-1 mRNA are constitutively expressed in the adult human heart. In the normal human ventricle, ALC-1 mRNA levels remained below a threshold level required for translation. Both HAND and ALC-1 mRNA were upregulated in a coordinated manner in the hypertrophied ventricle, eventually leading to ALC-1 translation. In the hypertrophied but not normal human ventricle, endogenous antisense ALC-1 mRNA could be involved in the post-translational expression regulation of ALC-1.

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