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Differential cooperation between dHAND and three different E-proteins☆

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Abstract

dHAND is a transcription factor belonging to the class B basic helix–loop–helix protein family and is expressed during embryogenesis in the heart, branchial arches, limb buds, and neural crest derivatives. Despite much study, the molecular mechanisms involved in the regulation of dHAND activity are not well understood. We therefore carried out yeast two-hybrid screening using full-length dHAND as bait, which led to identification of several dHAND-binding proteins, including three E-proteins: E2A, ME2, and ALF1. Subsequent analysis revealed that although their heterodimerization and transcriptional activities were similar, dHAND/E-protein heterodimers bind to an E-box element with differing affinities, suggesting they have distinct DNA binding specificities. Moreover, in situ hybridization showed that E-protein genes are expressed fairly ubiquitously among embryonic tissues, including the branchial arches and limb buds. By contrast, little signal was detected in the heart, suggesting that dHAND complexes with partners other than E-proteins in cardiac tissue.

Keywords: dHAND; E-protein; Basic helix–loop–helix; Transcription factor; Embryogenesis

The basic helix–loop–helix (bHLH) family of proteins is composed of two classes (A and B) of transcription factors that play important roles in a variety of biological processes, including cell growth and differentiation [1]. Class A bHLH transcription factors include the E2A, ME2 (E2-2), and ALF1 (HEB) gene products, also referred to as E-proteins [2–5]. These proteins are ubiquitously expressed in a wide range of cell types and function by forming homo- and heterodimers with other bHLH proteins [1]. Class B bHLH transcription factors, by contrast, are expressed in a tissue-specific manner and largely contribute to cell differentiation and tissue specification [1]. For example, the myogenic regulatory factors MyoD, myogenin, Myf5, and MRF4 belong to this subgroup and play key roles in skeletal myogenesis [6–8]. Class B bHLH proteins generally function as heterodimers with E-proteins and bind to the palindromic DNA sequence CANNTG (E-box motif) to regulate expression of their target genes [9–11].

The HAND proteins, dHAND and eHAND, are two class B bHLH transcription factors expressed in the heart, neural crest-derivatives, and extraembryonic

☆ *Abbreviations:* bHLH, basic helix–loop–helix; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; MBP, maltose-binding protein.

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tissues during embryogenesis [12–15]. Gene targeting experiments have shown that each of the HAND proteins is essential for proper development, and that they have distinct functions. Whereas dHAND-null mice die on embryonic day (E)9.5 as a result of defects in cardiovascular development [12,13], eHAND-null mice have lethal defects in both early extraembryonic tissue and the cardiovascular system [14,15]. It is also known that during branchial arch development, dHAND is expressed under the control of the endothelin-1-Dlx6 signaling pathway and plays an essential role in the patterning and development of skeletal components [16–18], but neither the target genes of dHAND nor the regulatory mechanisms governing dHAND activity are well understood. With that as background, our aim was to obtain additional information about the molecular mechanisms underlying the function of dHAND during embryonic development by using yeast two-hybrid screening to identify proteins forming heterodimers with dHAND, and to then examine some of the characteristics of those heterodimers.

Materials and methods

Constructs. cDNAs encoding mouse dHAND, eHAND, E47, ME2, ALF1a, ALF1b, and MyoD were cloned by PCR using a mouse E10.5 cDNA library (Gibco-BRL) as a template. After they were sequenced, an *Eco*RI–*Not*I fragment containing the full-length cDNA was inserted into pGEX (Amersham–Pharmacia Biotech) or pMAL (New England Biolabs) for construction of GST and MBP fusion proteins, respectively. These cDNAs were also cloned into the mammalian expression vector pCEFL-AU5 [19], which attaches an AU5-tag at the N-terminus, or pCMV-DBD (Stratagene), which yields a fusion protein with the Gal4 DNA-binding domain. The *Eco*RI–*Not*I fragment was also cloned into pAS-2-1 (Clontech) for construction of Gal4-DBD-dHAND, which was used for yeast two-hybrid screening.

Yeast two-hybrid screening. Yeast two-hybrid screening (Match maker II (Clontech)) was carried out using full-length dHAND as bait and an E10.5 embryonic whole mouse cDNA library. Positive clones were selected with growth medium lacking leucine, tryptophan, and histidine, and by detection of β-galactosidase activity. DNA fragments of the gene in positive clones were amplified by PCR. TA-cloning (Invitrogen) was then carried out followed by sequencing.

Cell culture and preparation of cell lysates. 293T cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37 °C under a 5% CO_2 atmosphere. C2C12 myoblastic cells were cultured in DMEM supplemented with 20% FBS and penicillin/streptomycin at 37 °C under a 5% CO_2 atmosphere. Cells were lysed in PBS containing 1% Triton X-100 and protease inhibitors, after which the crude lysates were cleared by centrifugation at 4 °C, and the supernatants were used for gel shift analyses and pull-down assays.

Preparation of GST and MBP fusion proteins. *Escherichia coli* (*E. coli*) (DH5α) transformed with pGEX, pMAL or their derivatives were grown in the presence of 0.4 mM IPTG for 3 h at 37 °C. The cells were then chilled on ice, centrifuged at 4 °C, and suspended in lysis buffer (PBS containing 1% Triton X-100 and 1 mM PMSF). After sonication, crude cell lysates were centrifuged at 4 °C. GST or MBP fusion proteins were purified from the resultant supernatants using GSH–Sepharose beads or amirose resin and eluted with 20 mM glutathione or 30 mM maltose, respectively.

Pull-down assays. GST-dHAND, GST-E-protein, MBP-dHAND or MBP-E-protein bound to beads was incubated for more than 2 h at 4 °C with cell lysates from 293T cells expressing AU5 fusion proteins. The beads were then washed three times with PBS containing 1% Triton X-100 and protease inhibitors, after which the bound proteins were eluted by adding 2.5× sample buffer and boiling for 5 min. These samples were then subjected to 10% SDS–PAGE, and the resolved proteins were detected by Western blotting using an anti-AU5 monoclonal antibody.

Western blotting and antibodies. For Western blotting, proteins separated by SDS–PAGE were transferred to a nylon membrane, which was then blocked in 5% skim milk prior to incubation first with anti-AU5 (BABCO) monoclonal antibody and then with anti-mouse IgG-conjugated horseradish peroxidase (ICN). The protein bands were visualized using an ECL Western Blotting Detection System (Amersham).

Gel shift analysis. Double-stranded oligonucleotide probes were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The binding sequence of the Thing1 (eHAND)/E47 heterodimer reported previously [20] was used as a probe. Specifically, the sequences of the probes used were: wild-type (E′),

5′-TTTGCAAGGGG**CA**TC**TG**GCATTCGCCC-3′and
5′-GGGGCGAATGCCAGATGCCCCTTGCAA-3′; mutant (mE′),
5′-TTTGCAAGGGGCGAACAGCATTCGCCC-3′ and
5′-GGGGCGAATGCTGTTCGCCCCTTGCAA-3′.

Bold letters indicate the E-box motif **CA**N**TG**. The reaction mixture (20 μl) consisted of 30 mM Tris–HCl (pH 7.5), 30 mM KCl, 0.6 mM EDTA, 0.6 mM DTT, 12% glycerol, 1 μg poly(dI–dC), 4 μl cell lysate, and 2 μl of labeled probe. The binding reaction was carried out for 30 min at room temperature, after which the samples were applied to 4% native PAGE. After the gels had dried, shifted bands were detected by autoradiography.

Transfection and reporter assays. The Gal4-DBD or Gal4-DBD construct was transfected into 293T or C2C12 cells along with pFR-Luc (Stratagene) and pRL-SV40 (Promega) using Lipofectamine Plus reagent (Life Technologies) according to the manual provided. The total amount of DNA used in each sample was adjusted to 0.3 μg. Forty-eight hours after transfection, luciferase activity was measured using a luminometer. Transfection efficiency was normalized on the basis of *Renilla* luciferase activity.

In situ hybridization. E10-E11 mouse embryos were fixed for more than 2 h in 4% paraformaldehyde, after which in situ hybridization was carried out as described previously [21]. The cDNAs used to generate antisense riboprobes were: a 700-bp mouse E2A, a 700-bp mouse ME2, a 640-bp mouse ALF1, and a full-length dHAND. RNA probes were synthesized using T3 or T7 RNA polymerase and labeled with digoxigenin.

Results

Screening for dHAND binding proteins

To identify proteins that interact with dHAND, we carried out yeast two-hybrid screening of a mouse E10.5 whole embryo cDNA library using full-length dHAND as bait. About 10^6 clones were screened, of which 552 grew in selection medium lacking leucine, tryptophan, and histidine (Table 1). Although they occurred with greatly varying frequency, three E-protein

Table 1
Clones obtained from yeast two-hybrid screening using full-length dHAND as bait

Genes	Number of clones (%)
E2A	245 (44.4)
ME2	88 (15.9)
ALF1	5 (0.9)
Known genes other than E-proteins	12 (2.2)
Unidentified genes	141 (25.5)
False positive	61 (11.1)

genes, E2A, ME2, and ALF1, accounted for more than 60% of the positive clones (Table 1).

E-proteins are assumed to be ubiquitously expressed and to have similar characteristics. In fact, however, little is known about their heterodimerization partners among class B bHLH proteins or their expression patterns during embryogenesis. We therefore aimed to analyze and compare the heterodimerization, DNA-binding, and transcriptional activity of these three E-proteins.

The effect of E47, ME2, and ALF1 on the transcriptional activity of dHAND

We initially evaluated the effects of each E-protein on the transcriptional activity of dHAND using reporter assays with a Gal4 fusion system. dHAND was fused to the Gal4 DNA-binding domain (DBD) at its N-terminus, after which the transcriptional activity induced by Gal4-DBD-dHAND was monitored as a function of luciferase activity. Gal4-DBD or Gal4-DBD-dHAND were transfected into C2C12 cells together with pFR-Luc, which contains the luciferase gene driven by the 5× Gal4 binding element. As shown in Fig. 1A, expression of Gal4-DBD-dHAND significantly increased (~8-fold) transcriptional activity, as compared with Gal4-DBD alone. Moreover, co-transfection of an AU5-tagged E-protein with Gal4-DBD-dHAND enhanced the transcriptional activity of dHAND an additional 2- to 4-fold, but had no effect on the luciferase activity stimulated by Gal4-DBD (Fig.1A).

We next compared the transcriptional activity of each E-protein fused to Gal4-DBD. We found that all of the Gal4-DBD-E-protein constructs elicited much higher levels of luciferase activity than Gal4-DBD alone (Fig. 1B). Although co-expression of E-proteins did not significantly affect the transcriptional activities of Gal4-DBD E-proteins, their transcriptional activities were markedly enhanced by co-expression with a class B bHLH protein (dHAND, eHAND or MyoD) (Fig. 1B). Thus, the transcriptional activities of heterodimers composed of a class B bHLH protein and an E-protein were substantially greater than those of either homo- or heterodimers made up of only E-proteins.

Formation of dHAND/E-protein dimers

We next carried out a set of GST and MBP pull-down assays to obtain information about dHAND/E-protein dimerization. After GST-fused dHAND and MBP-fused

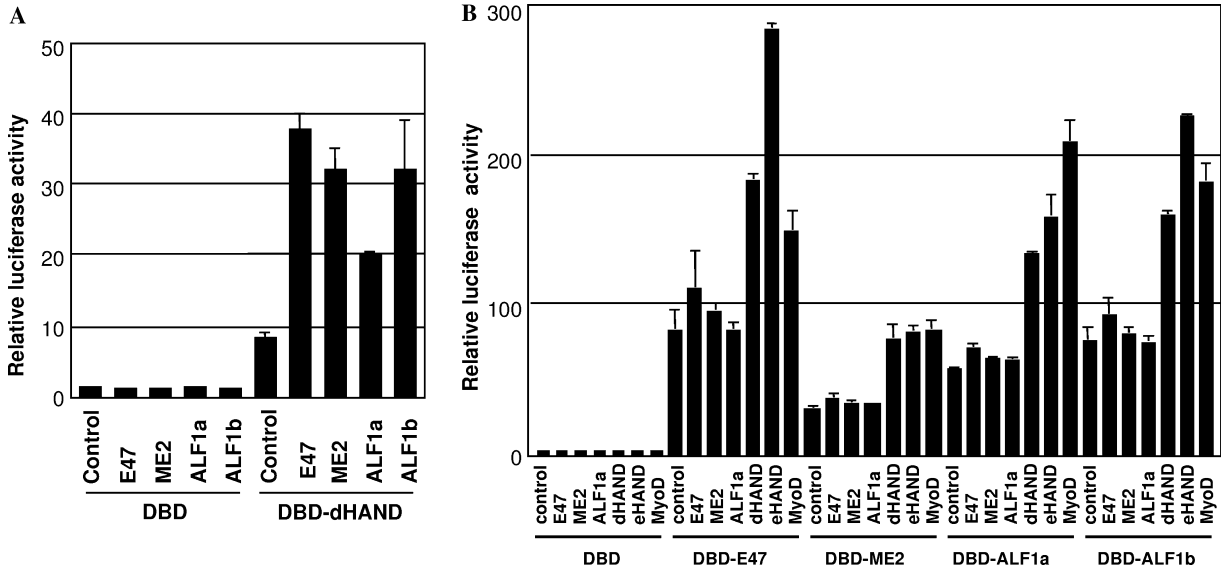

Fig. 1. Estimation of the transcriptional activity of dHAND. Gal4-DBD-dHAND, AU5-tagged expression plasmids, and a reporter plasmid containing the luciferase gene driven by the 5× Gal4 binding site were transfected into C2C12 cells. Forty-eight hours after transfection, cell lysates were prepared and luciferase activity was estimated. All assays were carried out in triplicate. (A) Effects of the indicated E-proteins on the transcriptional activity of DBD-dHAND. (B) Transcriptional activity of homo- and heterodimers composed of the indicated DBD-E-protein and an E-protein or a class B bHLH protein.

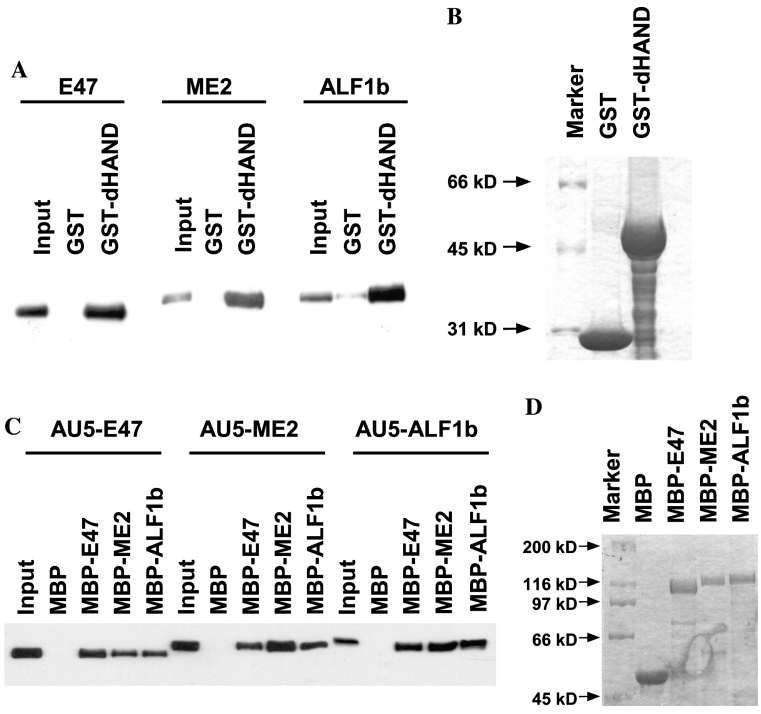

Fig. 2. Analysis of the interaction between dHAND and E-proteins. (A,C) GST-dHAND bound to GSH-beads (A) or MBP-E-protein bound to amirose resin (C) was incubated with lysate from 293T cells expressing AU5-E-proteins, after which the proteins were eluted and subjected to SDS–PAGE and Western blotting using an anti-AU5 monoclonal antibody. (B,D) GST and GST-dHAND (B) or MBP and each MBP-E-protein (D) used in the pull-down assays were boiled with SDS, subjected to SDS–PAGE, and stained with Coomassie brilliant blue.

E-proteins were expressed in *E. coli* and purified using GSH-beads or amirose resin, respectively, the bound protein was incubated with lysates from 293T cells expressing one of the AU5-E-proteins. After washing, the trapped proteins were eluted, separated by 10% SDS–PAGE, and detected by Western blotting using anti-AU5 monoclonal antibody. We found that AU5-E47, AU5-ME2, and AU5-ALF1b all bound to GST-dHAND, but not to GST alone (Fig. 2A). We then estimated homo- and heterodimer formation among E-proteins using the same assay and found that all three AU5-E-proteins bound to each MBP-E-protein but not to MBP alone (Fig. 3C). It thus appears that that all three E-proteins tested can form heterodimers with dHAND, as well as homo- and heterodimers among themselves.

The samples of GST, MBP, and their derivatives used in these assays are shown in Figs. 2B and D.

DNA-binding activity of each E-protein and dHAND/E-protein heterodimer

We used gel shift analyses to characterize the DNA binding activity of the dHAND/E-protein heterodimers. A ^{32}P-labeled oligonucleotide probe containing an E-box motif (**CANNTG**) was incubated with lysate from 293T cells expressing Myc-dHAND or one of the AU5-E-proteins. After electrophoresis, shifted bands were detected autoradiographically. Although no DNA binding to the probe was detected in the control and dHAND lysates, a clearly shifted band was detected in the E47 lysates, and that plus a second shifted band was detected in lysate containing both E47 and dHAND (Fig. 3A). Moreover, subsequent addition of anti-Myc or anti-AU5 antibody induced supershifts, confirming that each band was composed of dHAND and/or E-proteins (Fig. 3B). Although the dHAND/ME2 heterodimer also bound to this probe, the ME2, and ALF1b homodimers and the dHAND/ALF1b heterodimer did not (Fig. 3A). Furthermore, the shifted bands were specific for the E-box sequence, as no DNA binding was detected using a probe in which the E-box motif was disrupted by mutation (Fig. 3A). The levels of E47, ME2, and ALF1b protein in the cell lysates are shown in Fig. 3C. Taken together, these results indicate that E47, ME2, and ALF1b each bind to probe E′ with distinct characteristics, presumably reflecting the specificities governing formation of homo- or heterodimers among themselves or heterodimers with dHAND.

Patterns of E-protein gene expression during embryogenesis

The expression patterns of E-protein genes during embryogenesis have not been studied in detail and thus the partners of dHAND in specific tissues have not yet been identified. To obtain information about dHAND's partners, we carried out whole mount in situ hybridization and compared the patterns of expression of E2A,

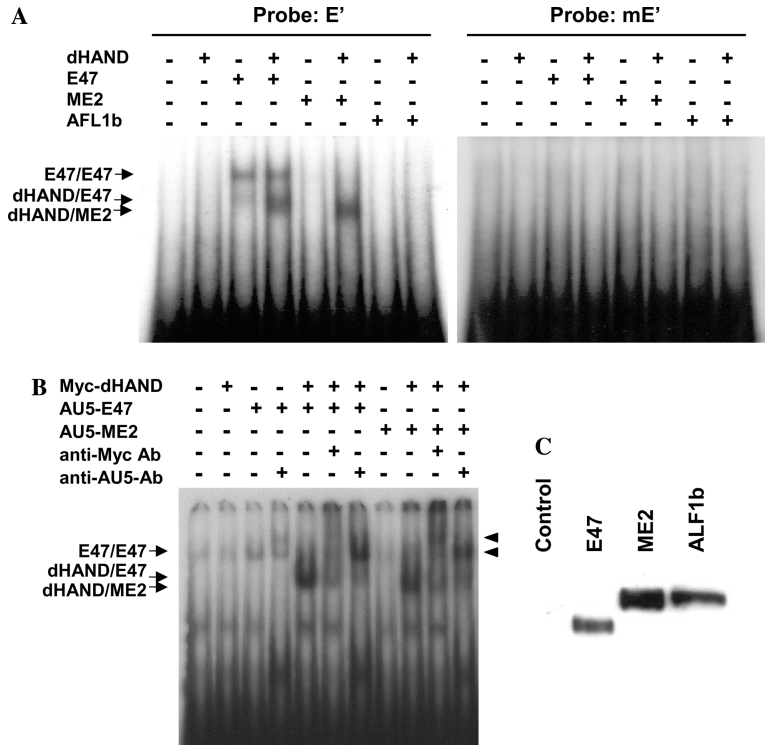

Fig. 3. DNA binding activity of dHAND/E-protein heterodimers. (A) Specific binding of dHAND/E-protein heterodimers to the E-box element. Lysate from 293T cells expressing Myc-dHAND or one of the indicated AU5-E-proteins was incubated with a ^{32}P-labeled oligonucleotide probe containing an E-box motif (E′) or a mutant probe (mE′), after which samples were subjected to 4% native PAGE followed by autoradiography. The positions of the E47 homodimer, dHAND/E47 heterodimer, and dHAND/ME2 heterodimer are shown on the left. (B) Supershift analysis. Treating the reaction mixtures with anti-Myc or anti-AU5 antibody produced supershifted complexes (arrowheads). (C) Western blots probed with anti-AU5-antibody showing E-protein samples used in this experiment.

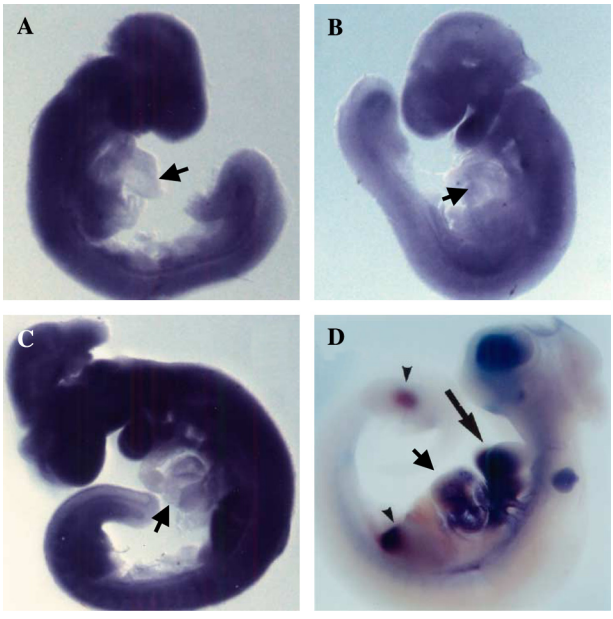

Fig. 4. E-protein and dHAND expression during embryogenesis. Whole mount in situ hybridizations with probes for E2A (A), ME2 (B), ALF1 (C), and dHAND (D) were carried out using E9.5 (A,B), E10 (C), and E10.5 (D) mouse embryos: short arrows, heart (A–D); long arrow, branchial arches (D); and arrowheads, limb buds (D).

ME2, and ALF1 in E10–E11 mouse embryos. As shown in Figs. 4A–C, all three E-protein genes were expressed fairly ubiquitously, though the signals for all three were comparatively weak in the heart. On the other hand, dHAND was strongly expressed in the heart, branchial arches, and limb buds (Fig. 4D), which suggests that dHAND/E2A, /ME2, and /ALF1 are readily formed in the branchial arches and limb buds, but that partners other than E-proteins are necessary for dHAND activity in the heart.

Discussion

In the present study, we used a yeast two-hybrid system to identify three dHAND-binding proteins: E2A, ME2, and ALF1. Although all three had similar transcriptional and dimerization activities, each dimer showed distinct binding activity to a DNA probe containing an E-box motif (CATCTG). We also showed that the expression of all three E-proteins was fairly ubiquitous during embryonic development, but that expression levels in the heart were quite low.

Recently, Dai and Cserjesi [22] reported that dHAND complexes with an E-protein to preferentially

bind a subset of E-box (CATCTG) probes, and that the sequences flanking the E-box motif are also involved in distinguishing dHAND-specific binding sites and are thus critical for its DNA binding specificity. Our present data are consistent with those findings, as the binding preferences of dHAND/E47 and dHAND/ME2 differed from those of dHAND/ALF1b: only dHAND/ALF1b did not bind our E-box probe containing a CATCTG motif. This suggests that each of the tested E-proteins may confer different DNA binding specificities upon dHAND, so that dHAND/ALF1b may bind to a target element other than CATCTG or may require a different flanking sequence to bind the same core motif. Taken together with the results of gel shift analyses showing there are differences in the DNA binding specificities of E-protein homodimers, these results suggest that the DNA binding specificity of dHAND reflects the structural differences of its E-protein partners, a situation that might also affect the activities of other class B bHLH proteins.

The patterns of E-protein expression during embryonic development were analyzed using whole mount in situ hybridization. As mentioned above, all three E-protein genes studied were expressed fairly ubiquitously but little signal was detected in the heart. E-protein expression appears to overlap that of dHAND in the branchial arches and limb buds, but not in the heart, which suggests that dHAND/E47 (E12), /ME2, and /ALF1 are formed in the branchial arches and limb buds, but that partners other than E-proteins may be necessary dHAND activity in the heart. At present, we do not know the heterodimerization partner(s) of dHAND in the heart, though one possibility is that it interacts with one or more of the HRT family proteins, which are bHLH proteins known to be highly expressed in heart [23]. Consistent with that idea, Firulli et al. [24] reported that dHAND binds directly to HRT in vitro. Another possibility is that dHAND forms an active homodimer in heart [22,24]. In the present study, however, we detected no dHAND homodimers in yeast two-hybrid assays, mammalian two-hybrid assays or gel shift analyses (data not shown). In addition, Dai et al. [25] recently showed that dHAND directly interacts with GATA4 via its C-terminal zinc finger domain to synergistically activate cardiomyocyte-specific promoters. This transcriptional synergy is dependent on a GATA site and association with p300, but is E-box-independent [25].

Finally, based on the present findings and those of others, we constructed a simple scheme to begin to conceptualize the molecular mechanisms involved in the regulation of dHAND function (Fig. 5). In the branchial arches and limb buds, dHAND binds to E2A, ME2, and ALF1 to form heterodimers with distinct DNA binding specificities and target genes. In the heart, by contrast, dHAND apparently acts by forming a homodimer or a heterodimer with one or more partners other than E-proteins. Given these characteristics, we anticipate that co-expression of various potential heterodimerization partners will enable determination of tissue-specific downstream targets of dHAND.

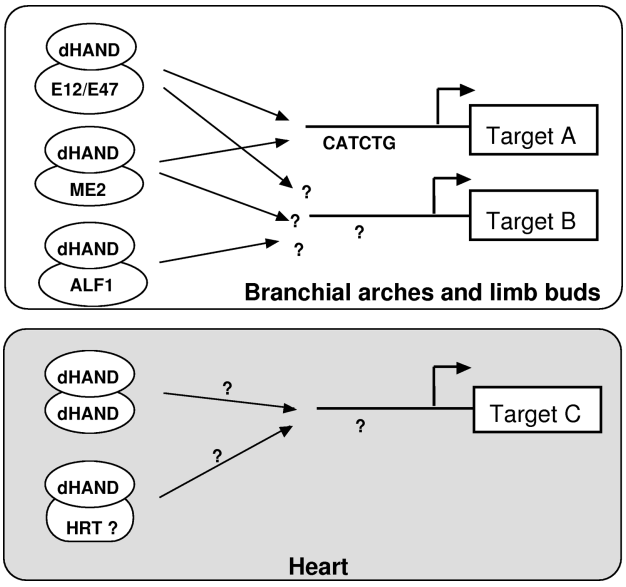

Fig. 5. Schematic diagram illustrating possible mechanisms involved in the regulation of dHAND activity in the heart or other tissues.

Acknowledgments

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