The Basic Helix-Loop-Helix Factor, HAND2, Functions as a Transcriptional Activator by Binding to E-boxes as a Heterodimer*

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HAND2 (dHAND) is a basic helix-loop-helix (bHLH) transcription factor expressed in numerous tissues during development including the heart, limbs, and a subset of neural crest derivatives. Functional analysis has shown that HAND2 is involved in development of the branchial arches, heart, limb, vasculature, and nervous system. Although it is essential for development of numerous tissues, little is known about its mode of action. To this end, we have characterized HAND2 transcriptional regulatory mechanisms. Using mammalian one-hybrid analysis we show that HAND2 contains a strong transcriptional activation domain in the amino-terminal third of the protein. Like most tissue-restricted bHLH factors, HAND2 heterodimerizes with the broadly expressed bHLH factors, the E-proteins. We determined the consensus DNA binding site of HAND2 and show that HAND2 binds a subset of E-boxes as a heterodimer with E12. Yeast two-hybrid screening of a neuroblastoma cDNA library for HAND2-interacting proteins selected HAND2 and numerous additional members of the E-protein family. Although HAND2 homodimer formation was confirmed by in vitro analysis, HAND2 fails to homodimerize in a mammalian two-hybrid assay but demonstrates robust HAND2/E12 interaction. We conclude that HAND2 functions as a transcription activator by binding a subset of E-boxes as a heterodimer with E-proteins.

The basic helix-loop-helix (bHLH) transcription factor comprises a large family of genes that are expressed in a wide array of eukaryotic organisms from yeast to humans (1). They are most often associated with developmental events including lineage determination and differentiation and regulation of tissue-specific genes. Their roles in development and the mechanisms of bHLH protein function have been most extensively studied in the developing myogenic and neural lineages. In these lineages, bHLH factors are essential for all aspects of development from cellular determination through terminal differentiation (reviewed in Refs. 2 and 3).

The bHLH family is divided into several classes based on structure, function, and expression pattern during development (2). The distinguishing structural characteristics of this family of transcription factors are a DNA binding basic domain and a helix-loop-helix dimerization domain. In general, these factors regulate transcription through direct binding to DNA in the regulatory regions of genes. Binding of bHLH factors requires dimerization with other members of the bHLH family. Tissue-restricted bHLH factors compose the largest class and generally function by dimerization with a small but broadly expressed class of bHLH factors, the E-proteins. Members of the tissue-restricted class of bHLH factors act to regulate transcription as activators, repressors, or both (3). In addition, transcriptional activity of the tissue-restricted bHLH proteins requires association with other bHLH and non-bHLH proteins. For example, the myogenic and neurogenic factors regulate at least partially through direct interaction with histone acetyl transferase factors (4, 5). These interactions have been shown to be essential for modulating transcriptional activity as well as controlling differentiation (6, 7). Repression by bHLH factors also occurs through recruitment of repressor proteins. Among the best characterized of these repressors are members of the Enhancer of split/Hairy family of repressors (11, 12).

bHLH proteins most commonly bind a DNA motif called an E-box (CANNTG), first identified as an essential element in immunoglobulin heavy chain gene regulation (8). The E-box element has been identified as essential for the regulation of a wide array of genes in neurons, muscle and other tissues. Because of their low level of complexity, E-boxes are found in high numbers throughout the genome; however, the central two bases and sequences flanking the E-box provide additional DNA binding specificity.

Within the tissue-restricted class of bHLH factors, groups of proteins share similarities in sequence and expression patterns. Although members of these families often share overlapping expression patterns, they have different functions. For example, members of the twist-related bHLH subfamily that includes twist (9), Dermo-1 (10), scleraxis (11), paraxis (12), HAND1 (13–15), and HAND2 (16) are all expressed in mesodermal derivatives. Gene targeting analysis has shown that these bHLHs affect development of overlapping and distinct mesodermal lineages. Although expressed predominantly in mesodermally derived tissues, they can also function to affect development and tissue-specific gene expression in other lineages.

The two HAND bHLH transcription factors were cloned in...
several laboratories and are called variously eHAND/dHAND (13, 16), Th/HTh (15), and ext/ed (14). During embryogenesis they are expressed in numerous tissues including the heart, neural crest derivatives, smooth muscle, and extramembrane lineages, suggesting that they play a role in the development of both mesodermal and nonmesodermal lineages. The expression patterns of the HAND genes is reflected in the distinct phenotypes produced by gene ablation through site-directed mutagenesis (17–19).\(^2\) HAND1 null mice have extensive defects in the development of extramembrane lineages leading to early embryonic lethality, whereas HAND2 null embryos succumb to cardiovascular defects.

Although the biological importance of HAND2 during development has been well established, the molecular mechanism of HAND2 function has not been examined extensively. It has been shown that HAND1 and HAND2 can interact to form a heterodimer and unexpectedly that HAND2 also homodimerizes (20). HAND1 and HAND2 contain several regions of high sequence similarity including almost complete identity in the HLH region, suggesting the two proteins share some common functions. However, in their basic domain they share only 62% overall identity and no identity in the core amino acids identified as essential for DNA sequence recognition in a number of bHLH factors. This suggests that HAND2 binds a different DNA sequence and regulates different target genes.

To better understand the differences between these proteins and their molecular bases of action, we have undertaken an analysis of HAND2 activity. We find that HAND2 activates gene expression through a transcriptional activation domain located in the amino-terminal region that can be masked by intra- or intermolecular interactions. Using cyclic amplification and selection of targets (CASTing) (21), we determined that HAND2 binds an E-box distinct from that bound by HAND1. E-box-specific binding is dependent on heterodimer formation with E-proteins. We show that the consensus sequence for this E-box supports HAND2 dependent transcription in vivo. Our analysis suggests that HAND2 forms a homodimer in vitro but functions as a heterodimer with E-proteins in vivo and functions to activate transcription through binding a subset of E-boxes as a heterodimer with the broadly expressed E-proteins.

**EXPERIMENTAL PROCEDURES**

**Plasmids—** The following constructs were generated. The original HAND2 cDNA clone was obtained from a Plox mouse embryonic cDNA library. For pBSKII-H2–7, the 1.2-kb CDNA insert was subcloned as an EcoRI and HindIII fragment into Bluescript vector (Stratagene). For pSET2b-His-HAND2, the coding region of HAND2 was amplified by PCR from pBSKII-H2–7 using the primers 5′-GAGGCCGGCCATGGGTCCTTGCGG-3′ and 5′-TCTGGCCGCGGCCTCCCGC-3′ to simultaneously add NcoI restriction sites at the start or translation and after the stop codon. The PCR product was cloned into the NcoI site of pSET2b (Invitrogen). For pGEX2TK-GST-dHAND2, the NcoI fragment of HAND2 from pSET2b was end-filled with Klenow and cloned into the Smal site of the pGEX2TK (Amersham Biosciences). For pcDNA-Hs-HAND2, the BamHI and EcoRI fragment from pSET2B-HAND2 was cloned into BamHI and EcoRI sites in pcDNA-Hs B (Invitrogen). For pAS2-HAND2, the NcoI HAND2 fragment from pSET2b-HAND2 was cloned into the NcoI site of pAS2 (gift of Steve Ellidge, Baylor College of Medicine). For pSG424-HAND2, HAND2 was excised from pSET2b-HAND2 as NcoI fragment, end-filled with Klenow, and cloned into pSG424-Gal4 vector at the Smal site fusing HAND2 in-frame with the DNA binding domain (DBD) of Gal4.

The generation of amino-terminal deletions of HAND2 was by PCR synthesis using pSG424-HAND2 as template. Synthesized fragments were cloned into the EcoRI-XhoI sites of the expression vector pM (CLONTECH). The sense primer (S) for AA 1–20, 1–40, 1–60 was located in the Gal4-DBD binding domain, 5′-CATGAATAAGTGCCGA-CATCA-3′. The antisense (AS) primers were: AA 1–20, 5′-GAGCTGTCATAGATGGTGATCGCTAGG-3′; AA 1–40, 5′-GGGCTTCTAGAGAGGGGAGTCTTCTTGTT-3′; AA 1–60, 5′-GGGCTTCTAGAGAGGAGTCTTCTTGTT-3′. For pSG424-HAND2, the affinity tagenesis (17) was 5′-GATTAGAATTTCGACTTACCATCGGCGACG (S) and 5′-GAAATCTTCAAGGCGGCGACATTCCCTCCAATAGT-3′ (AS). For AA 40–65, the S primer was the same that used for deletion AA 40–85, and the AS primer was the same as used for AA 51–73 synthesis.

For pSG424-E12(bHLH), the bHLH domain of E12 fused to the Gal4 DNA binding domain was a gift from Anthony Filitti (20). For pRSV-R2b, the HindIII/BamHI fragment of HAND2 from pcDNA-Hs-HAND2 was cloned into pRcR2b (Invitrogen). For 4HE-TK-CAT, the reporter contains four copies of the HAND2 consensus binding site E-box (CTCAGACGGCACCCGTCACT), cloned up-stream of a minimal TATA-thymidine kinase promoter. For TATA-Luc, the rat a-miosyn TATA box (5′-AGCCTTCGAGCATATAAGG-3′) was cloned into the HindIII site of pG2L basic (Promega) (22). For 6HE-a-miosyn-Luc, six copies of the HAND2 E-box were cloned up-stream of the TATA box of pTATA-Luc.

**Antibody Production—** HAND2-specific antibodies were generated in rabbits against a synthetic peptide (VKEERKKELNEILK) corresponding to a region in the amino-terminal portion of the protein using standard procedures (Research Genetics). Rabbit serum was tested for specificity by Western blot analysis on bacterially produced HAND1 and HAND2 protein. The serum was further tested by expression of HAND1 or HAND2 in NIH3T3 cells by transient transfection and testing for specificity by immunocytochemical analysis and by Western blot analysis of extracted cellular proteins.

**DNA Binding Site Selection—** The consensus DNA binding site was cloned in vitro using a modified CASTing protocol (21). A 63-bp oligonucleotide containing 16 random bases flanked by EcoRI and HinDIII sites (5′-AGCTTCGTGCAGTATAAAGGG-3′) was synthesized and made double-stranded by PCR with the primer (VB2B)-AAAAACATATGGCATGAGGA using Vent DNA polymerase (New England Biolabs). 5 μg of double-stranded DNA was mixed with 2 μl of each in vitro TNT translated HAND2 and E12 protein in a 20-μl binding reaction mixture containing 50 mM Tris-HCl (pH 7.6), 80 mM NaCl, 10 mM MgCl₂, and 0.5 μg Poly(dI:dC). After a 30-min incubation, 0.5 μl of rabbit anti-HAND2 antibody No. 13 was added, and the mixture incubated for an additional 1 h at room temperature. HAND complexes were pulled down with the addition of 10 μl of protein A beads at 4 °C for 1 h, and the mixture was pelleted and washed three times with Tris-Cl (pH 7.6), 80 mM NaCl, and 0.1% Nonidet P-40. The DNA was eluted by resuspending the beads in 40 μl of PCR buffer, boiled for 5 min, and cleared by centrifugation at 12,000 g for 1 min. PCR amplification of the eluted DNA was performed at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 45 s, for 20 cycles using 15 pmol of primers VB2B and VB3 (TGTAAGACCCGAGCCGAGGA). Half of the reaction was analyzed by gel electrophoresis, and the remainder was extracted once with phenol/chloroform and ethanol-precipitated for the next CASTing cycle. After five rounds of selection, the amplified PCR product was end-labeled with 32P and further selected by gel mobility shift assay. The HAND2 gel shift complex was excluded from the gel, eluted and re-amplified by PCR, and cloned into the EcoRI-HindIII sites of pBS-KSII (Stratagene) for sequencing.

**In Vitro Translation—** HAND2 and E12 proteins were synthesized in vitro using the TNT-coupled Rabbit Reticulocyte Lysate system (Promega) using T7 RNA polymerase transcription of pSET2b-His-HAND2 and pB8-E12. The size of the translated proteins was confirmed by SDS-PAGE using 35S-labeled proteins synthesized in parallel.

**Bacterial Expression of HAND2—** The expression vector for glutathione-S-transferase (GST)-HAND2 fusion protein, pGEX2TK-HAND2, was cloned into JM109 to produce purified GST fusion proteins. 50 μl of an overnight culture of JM109 cells containing a GST fusion protein vector was diluted 1:10 with Luria broth containing 100 μg of ampicillin/ml and cultured in a rotary shaker until the optical density at 600 nm reached 0.6–1.0. Next, expression of the GST-HAND2 fusion protein was induced for 3 h by the addition of isopropyl-\(\beta\)-thiogalactopyranoside to a final concentration of 100 μM. Cells were harvested by centrifugation and the pellet resuspended in 5 ml of

\(^2\) P. Cserjesi, unpublished observations.
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ice-cold MTPBS buffer (150 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4, pH 7.3). The cell suspension was sonicated four times for 15 s, and then Triton X-100 was added to a concentration of 1% and the suspension incubated for 1 h at room temperature. The lysate was centrifuged at 10,000 × g, 4 °C. The supernatant was mixed with 2.5 ml of glutathione-Sepharose 4B beads (pre-equilibrated with MTPBS buffer) and incubated at 4 °C for 2 h while shaking. The beads were then centrifuged through 20% sucrose in MTPBS for 5 min at 2,500 rpm, washed once each with MTPBS buffer, 50 mM Tris, pH 8.5, 150 mM NaCl, and 50 mM Tris, pH 8.5, 150 mM NaCl, 2.5 mM CaCl2. The GST fusion protein was then eluted from the Sepharose 4B-glutathione beads with 25 mM glutathione.

Analysis of the in Vitro Interaction between HAND2 and E12 Proteins—One microgram of bacterial produced GST-HAND2 fusion protein was conjugated to Glutathione-beads, mixed with 5 μl of [35S]-labeled proteins in 250 μl of binding buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.25% Nonidet P-40), and incubated at 4 °C for 2 h with gentle rocking. The complexes were washed three times in 1 ml of binding buffer. As a negative control, the [35S]-labeled proteins were incubated with GST-beads alone. The beads were heated to 95 °C for 5 min in SDS sample buffer and interacting proteins analyzed by SDS-PAGE followed by autoradiography.

Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotide was annealed and end-radio labeled with 50 μCi of [32P]-dCTP using Klenow to fill in protruding ends. For each EMSA assay, 2 μl of each in vitro translated protein lysate was combined and preincubated for 30 min at 37 °C prior to the addition of EMSA binding buffer and oligonucleotides. The protein mixture was incubated in binding buffer (50 mM Tris-HCl (pH 7.6), 80 mM NaCl, 8% glycerol, and 0.25 μg poly(dI:dC)) at room temperature for 20 min; ~0.1 pmol of radiolabeled oligonucleotide probe was added and incubated 30 min at room temperature in a 10-μl total volume. The incubated mixture was separated on a 5% native polyacrylamide gel run buffered with 0.5× Tris borate-EDTA. The gel was dried and exposed to x-ray film for autoradiographic analysis.

Transfection—Cells were plated to 20–40% confluence 12–18 h prior to transfection. Cells were transfected by calcium phosphate precipitation. Transfection was performed at a reporter to activator ratio of 1:2. Cells were harvested 48 h post-transfection, and lysates were assayed for CAT activity and normalized for transfection and assay efficiency using mRNA isolated from neuro-2a neuroblastoma cells by oligo d(T) cellulose chromatography. The library was constructed in the pGAD10 yeast expression vector with a yeast two-hybrid cDNA construction kit (CLONTECH). First strand cDNA was synthesized using both random and oligo-d(T) primers. Prior to cloning, double-stranded cDNA was size-selected for molecules >500 bp. Analysis of 20 randomly selected clones from the library showed an average size of 1.2 kb with 95% of the clones containing an insert.

The HAND2 coding region was inserted in-frame with the GAL4 DNA binding domain (AA 1–147) into the yeast expression vector pAS2. This construct was transformed into the yeast strain Y190 and expression of HAND2 analyzed by Western blotting with HAND2-specific antibody. The neuro-2a library was transformed into yeast expressing HAND2, and cells were plated on SD/His/Leu/Tre to select for colonies containing interacting proteins. Plasmids from positive colonies were transfected into a leuB HB101 strain of Escherichia coli by electroporation. DNA from 80 positive clones was sequenced and the sequences analyzed for homology to known clones using GenBank™.

**RESULTS**

HAND2 Contains a Strong Activation Domain Located in the Amino-terminal Region—The demonstrated ability of HAND2 to regulate developmental events suggests that like other bHLH factors, HAND2 regulates gene transcription. To analyze the mechanism by which HAND2 regulates gene expression, we examined the ability of HAND2 to stimulate transcription using a mammalian one-hybrid assay. HAND2 was fused with the yeast Gal4 minimal DNA binding domain to create a chimeric transcription factor dependent on HAND2 for transcriptional activation but not DNA binding. The Gal4-HAND2 fusion plasmid and a luciferase reporter plasmid containing five Gal4 DNA binding sites upstream of the E1b basal promoter were co-transfected into COS-7 cells. Because the activation domains of numerous bHLH transcription factors including MyoD (23), Mash1 (24), and E12 (25) are regulated through intramolecular interactions, we generated a series of HAND2 deletions fused to Gal4-DBD and tested their ability to activate transcription by transient co-transfection with the 5E1b-Luc reporter construct (Fig. 1A). We found that full-length HAND2 does not significantly activate transcription. The construct containing the bHLH plus the carboxyl portion of HAND2 (encoding AA 86–217) activates transcription to slightly higher level than the full-length HAND2 protein. The Gal4 fusion construct containing only the amino-terminal region of HAND2 (encoding AA 1–85) activates transcription 47-fold higher than the full-length HAND2 protein. These results suggest that HAND2 functions as a transcriptional activator. The high activity associated with the amino-terminal domain and low activity observed for the full-length protein suggest that HAND2 function is regulated by intra- or intermolecular interactions that mask full transcriptional activity.

To further delineate the amino-terminal transcriptional activation domain, we generated a series of deletions within the HAND2 amino-terminal region (Fig. 1B). Deletions were generated within the first 85 amino acids of by PCR, and the
resulting sequence was cloned in-frame to the GAL4-DBD. These deletion constructs were tested by co-transfection into COS-7 cells with the Gal4-luciferase reporter, pG5-E1b-Luc. Deletion of amino acids 61–85 resulted in a 90% reduction in transcriptional activity suggesting that this region is essential for activation. Deletion of the first 40 amino acids of HAND2 reduced activity by only 20%, whereas constructs containing only the first 40 amino acids weakly activate transcription. All deletions within amino acids 41–85 reduced transcriptional activity. Taken together, the deletion analysis suggests that a core activation domain is situated between amino acids 41–85.

The masking of transcriptional activity in full-length HAND2 was observed in a number of other mammalian cell lines (data not shown) as well as in a yeast two-hybrid screen. To examine whether the failure of full-length HAND2 to activate transcription in yeast was due to a masking effect, as seen in mammalian cells, deletions of HAND2 were subcloned into the yeast Gal4 expression vector pAS2 and tested for transcriptional activity (Table I). As in mammalian cells, full-length HAND2 protein, the bHLH and carboxyl-terminal regions (86–217), and the carboxyl-terminal region alone (166–217) all failed to activate transcription in yeast. However, as in mammalian cells, the amino-terminal region of HAND2 robustly activated transcription in yeast. The activity of the transcriptional activation domain and the masking by the rest of the protein is conserved in both yeast and mammalian cells. These results suggest that a strong transcriptional activation domain resides in the amino terminus of HAND2, and in both yeast and mammalian cells this domain is masked in the full-length protein.

**Interaction of HAND2 and E-proteins in Vivo and in Vitro—** The protein dimerization and DNA binding activities of bHLH proteins are separable events regulated by the HLH and basic domains, respectively. It has been reported that HAND2 homodimerizes and also heterodimerizes with HAND1 as well as interacting with E-proteins in vitro (20) (data not shown). However, the interaction between HAND1 and HAND2 proteins when tested for interaction in a mammalian two-hybrid assay was weak, suggesting that more complex interactions may be involved in regulating HAND protein interactions. We therefore screened a neuro-2a neuroblastoma cell line library for HAND2-interacting proteins. We screened ~6 × 10^6 clones using full-length HAND2 as bait in a yeast two-hybrid screen. From this screen, 80 HAND2-interacting clones tested positive in subsequent re-screens (Table II). Members of the E-protein family were the most highly represented class of HAND2-interacting proteins, and a number of different family members were represented, including E47, E12, and ITF2a.

It has been reported that HAND2 forms homodimers with HAND2 and heterodimerizes with HAND1 in yeast (20). Our screen confirmed that HAND2 forms homodimers, because HAND2 represented 14% of the clones isolated. Although HAND1 transcript is also expressed at levels comparable to HAND2 in neuro-2a cells (data not shown), we did not identify HAND1 in our yeast two-hybrid screen. Two other clones that were represented at high levels in our screen encoded nucleophasmin/B23 and CENP-B. Nucleophasmin/B23 is a nucleolar protein that has been implicated in regulating the cell cycle (26, 27). CENP-B is a centromeric protein implicated in chromosome segregation (28). These clones were not tested further. We isolated two clones encoding the protein JAB1. JAB1 was identified originally as a c-Jun- and JunD-interacting protein that potentiates DNA binding (29). More recently JAB1 was shown to regulate the cell cycle through an interaction with p27^kip1 (30).

**Interaction of HAND2 with E12—** Because the yeast two-hybrid screen yielded both a large number of E-proteins and HAND2 itself, it remains unclear whether HAND2 functions predominantly as a homo- or heterodimer. We further examined the ability of HAND2 to interact with itself or E-proteins. We compared the ability of in vitro synthesized HAND2 or E12 to interact with a GST-HAND2 fusion protein (Fig. 2A). Both HAND2 (lane 3) and E12 (lane 6) show an interaction with GST-HAND2 but not with GST alone (lanes 2 and 5). Because of the high sequence conservation within the helix-loop-helix domains of HAND1 and HAND2, we also tested the ability of HAND2 to heterodimerize with HAND1. As reported previously (20), HAND2 interacts with HAND1 in vitro (data not shown). These results confirm our yeast two-hybrid results and demonstrate a direct interaction between HAND2 and E12 and HAND2 with HAND2.

To determine whether these in vitro results reflect in vivo interactions of HAND2, we compared the effectiveness of HAND2/E12 and HAND2/HAND2 interactions using a mammalian two-hybrid assay. In this assay, the level of transcriptional activity is a measure of the relative strength of interaction between two proteins. In neuro-2a cells transfected with the Gal4-HAND2 construct, transcriptional activity of the Gal4-dependent reporter is enhanced by 12-fold over the Gal4 control (Fig. 2B). Transfection with Gal4-E12(bHLH) alone, which lacks an activation domain, does not activate the reporter to an appreciable level. When HAND2-VP16 and Gal4-E12(bHLH) are co-transfected, transcriptional activity is enhanced over 60-fold, whereas co-transfection of Gal4-HAND2 and HAND2-VP16 enhances transcription by only 8-fold. These results suggest that whereas HAND2 can form homodimers in vitro and in yeast, in mammalian cells the functional complex is preferentially a heterodimer with E-proteins.

**Identification of HAND2 High Affinity Binding Sites—** The preferential interaction of HAND2 with E-proteins in mammalian cells suggests that, like other tissue-specific bHLH factors, it regulates transcription through binding an E-box DNA binding site. However, the ability of HAND2 to directly bind DNA and regulate transcription has not been examined. To further analyze the role of HAND2 as a transcriptional regulator and allow future identification of HAND2 gene targets, we deter-

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**Table I**

**Transcriptional Properties of HAND2**

| Gal4-DBD-HAND2 constructs | β-galactosidase activity |
|---------------------------|-------------------------|
| HAND2 (1–217)             | 1                       |
| N terminus (1–85)         | 70                      |
| bHLHC (86–217)            | 1                       |
| C terminus (166–217)      | 1                       |

**Table II**

**Yeast two-hybrid analysis of a neuro-2a neuroblastoma cell library for HAND2-interacting proteins**

A yeast two-hybrid library was constructed using cDNA from the neuroblastoma cell line neuro-2a and screened with full-length HAND2 as bait. The different classes of clones obtained are represented as the percentage of 80 clones isolated.

| Yeast two-hybrid screen | % of clones |
|-------------------------|-------------|
| E-proteins              | 41          |
| HAND2                   | 14          |
| Nucleophasmin/B23       | 28          |
| Centromeric protein CENP-B | 14         |
| JAB1                    | 3           |
mined the high affinity DNA recognition sequence that interacts with HAND2/E12 heterodimers. We employed the highly sensitive and unbiased CASTing method to identify HAND2/E12 DNA binding sites (21). HAND2 and E12 proteins were synthesized in vitro using the TNT-coupled rabbit reticulocyte system. The size and concentration of the translated products were verified by SDS-PAGE analysis of co-translated 35S-labeled proteins (data not shown). An equimolar mixture of HAND2 and E12 protein was incubated with a mixture of 63 bp double-stranded oligonucleotides containing a tract of 16 random base pairs flanked by primer and cloning sites (21). The DNA-protein complexes containing HAND2 were immunoprecipitated with rabbit polyclonal anti-HAND2 antibody. The antibody is against a synthetic peptide generated from a sequence in the amino-terminal region of HAND2 (HPEMSPPDYSMALSYSPE). The immunoprecipitated DNA-protein complexes were purified, and the DNA was amplified by PCR. After five rounds of repeated selection and amplification the oligonucleotides were purified and labeled with 32P nucleotides and were further enriched for HAND2-containing complexes by gel mobility shift assay (Fig. 3A). In the presence of both HAND2 and E12 protein, a DNA-protein complex formed with the labeled oligonucleotides (Fig. 3A, lane 2). The complex is disrupted when the incubation mixture is preincubated with HAND2-specific antibodies prior to electrophoresis (Fig. 3A, lane 3). Incubation of the selected oligonucleotides with HAND2 protein alone did not generate a HAND2-dependent DNA-protein complex (Fig. 3A, lane 1), suggesting that HAND2/HAND2 are not the functional complexes.

To purify the selected oligonucleotides, the region containing the retarded HAND2/E12-DNA complex was excised, the DNA was eluted from the gel and PCR-amplified, and the products were cloned and sequenced. Of 55 sequenced clones, 36 clones contained an E-box sequence (Fig. 1B). Clones not containing an E-box motif were tested for binding to HAND2 and HAND2/E12 and shown not to bind (data not shown). HAND2 protein did not bind to representative E-boxes as a homodimer (data not shown). Most of the E-box containing sequences display a distinct bias in the choice of the middle two bases in the E-box and also in the choice of nucleotides flanking the E-box. The majority of HAND2 E-boxes carry a CATCTG core. However, like other bHLH transcription factors, HAND2 is promiscuous and also bound the E-boxes CATGTG, CACCTG, and CACGTG but at a lower frequency.

The nucleotide frequency at each position of the selected
HAND2 E-box sequences is summarized in Fig. 3C. The specificity of DNA binding by bHLH transcription factors is determined by both the core variable nucleotides within the E-box as well as the flanking sequences. The nucleotides flanking the HAND2-selected E-boxes are also highly conserved. The preferred 5′ flanking sequence is six Gs with a C immediately adjacent to the E-box. The preferred 3′ sequence contains a conserved G that is within the PCR primer sequence flanking the randomized 16-bp sequence. We show that this base is indeed a part of the preferred HAND2/E12 binding site in subsequent mutational analysis of the sequence (Fig. 4). A combination of a long 5′ preferred sequence in combination with this fixed G explains the high bias for the E-box being located at the 3′ end of the randomized sequences.

**HAND2/E12 Heterodimer Preferentially Binds the Extended HAND2 E-box**—The promiscuous nature of DNA binding by many bHLH transcription factors to E-boxes suggests that specificity depends in part on subtle differences in binding affinities. We investigated the requirement for key bases within the selected HAND2 consensus sequence for high affinity binding to the HAND2 selected E-boxes. Within the E-box sequence, the HAND2 CASTing selected four types of CANNTG core motifs: CATGTG, CACCTG, CATCTG, and CACGTG. Because HAND2 homodimers may bind to a small subset of E-boxes that were not detected in EMSA of the total selected oligonucleotides in Fig. 3A, these E-box sequences were tested for their ability to bind with HAND2, E12, and HAND2/E12 proteins by gel mobility shift assay (Fig. 4A). HAND2 is unable to bind these sites as a homodimer although the CACGTG sequence contains a dyad symmetry that may be expected to bind homodimers. The HAND2/E12 heterodimer binds three of the selected E-boxes robustly but binds the E-box CACCTG only weakly. The CACCTG motif has been shown to bind numerous bHLH factors including the MyoD family of bHLH factors (31) and neurogenic bHLH factor Mash1 (24). These results highlight the importance of the core E-box sequences in target selection by bHLH factors.

Conservation of the sequences flanking the HAND2/E12 selected E-box sequences suggests that HAND2/E12-specific binding is also affected at these positions. We tested the effect of mutating E-box flanking sequences by gel mobility shift assays (Fig. 4B). Most HAND2/E12-selected oligonucleotides contain a string of G residues at position −5 to −10 and all but one selected oligonucleotide contain a C at position −4 (Fig. 3C). All but two of the HAND2/E12-selected oligonucleotides contained a G at position +4. As mentioned above, the selected sequences were heavily biased toward one end of the randomized 16-bp selected sequence. The G at position +4 represents...
the first base of the constant region flanking the randomized nucleotides, and if it were a high affinity nucleotide it would explain the reason for the bias to this region of the probe. To test the requirement of a G at this position for HAND2/E12 binding, we examined the ability of HAND2/E12 to bind modified oligonucleotides. Changing position +4 to a C had little effect on HAND2 complex formation, but a change to an A reduced complex formation substantially. The results suggest that sequences both within and flanking the HAND2-selected E-box have an effect on complex formation. However, these experiments do not address the relative effect different base changes have on the binding affinity of HAND2/E12.

To test the quantitative effect of the core and flanking E-box nucleotides on HAND2 complex formation, we used competitive gel mobility shift assays (Fig. 4C). A 32P-labeled HAND2 E-box probe was incubated with HAND2/E12, and the protein-DNA complex was challenged with excess unlabeled oligonucleotides. Changes in the HAND2 E-box flanking sequences were tested for their ability to compete for the HAND2/E12 complex. Nucleotides upstream of the E-box are the most promiscuous. However, the G at position +4 is essential for high affinity binding. The relative effect of the changes was nucleotide-specific. Changing the G to a C at +4 mildly reduced the ability of the mutant oligonucleotides to compete for the HAND2 E-box but to a change to an A abolished all competition. The changes within the HAND2 E-box core consensus sequence had a dramatic effect on their ability to form HAND2 complexes. Changing the T at position −1 to either a G or a C severely inhibited HAND2 from binding the E-box (Fig. 4C). The specificity of HAND2 binding to a unique extended E-box suggests that during development it regulates a distinct subset of genes containing these sites.

**HAND2 Can Activate Transcription through the High Affinity HAND2 E-box Element Synergistically with E12**—Our *in vitro* results indicate that HAND2 binds to a distinct set of E-boxes as a heterodimer with E12. We next determined whether the high affinity HAND2 E-box functions *in vivo* to activate transcription in a HAND2-dependent manner. We generated a HAND2 E-box-dependent CAT reporter carrying four copies of the HAND2 E-box site upstream of a thymidine kinase basal promoter. Transient co-transfection of this reporter with a HAND2 expression construct in NIH3T3 cells enhanced transcription 17-fold relative to reporter alone (Fig. 5A). Because the transcriptional activation domain located in the amino third of HAND2 is masked in the context of the whole protein, we determined whether E12 is able to unmask this domain through dimerization. When HAND2 and E12 were co-transfected, the level of transcriptional activity was additive. E12 also activates transcription through this site but to a lesser extent than HAND2 (Fig. 5A). Because the addition of E12 did not increase transcription to the same extent observed with the amino terminus of HAND2 alone, our results suggest that E12 interaction with HAND2 alone cannot unmask the transcriptional activation domain of HAND2.

To examine HAND2 function in the context of a different promoter, we examined HAND2 regulation of transcription via six copies of the E-box upstream of a TATA box derived from the α-myosin heavy chain minimal promoter (Fig. 5B). A fixed concentration of reporter was co-transfected with increasing concentrations of HAND2 expression vector. The level of transcriptional activity increased in proportion to the amount of HAND2 vector transfected with a maximum at −1.6 μg of DNA/60-mm plate. The plateau at this concentration may reflect the limited availability of HAND2 dimerization partners. These results indicate that HAND2 can activate transcription in a dose-dependent manner via different promoters.

**DISCUSSION**

The bHLH family of transcription factors plays a key role in cell fate determination and differentiation during embryogenesis. For example, myogenesis and neurogenesis has been shown to depend on cascades of bHLH factors acting as transcriptional activators or repressors in a wide variety of organisms. Often, related bHLH factors cross-activate each other and autoregulate their own transcription. The molecular mechanisms underlying bHLH function in some cases has been examined in detail and shown to involve multiple levels of regulation including protein/protein interactions (2). The twist family of bHLH factors is found in a wide variety of organisms from nematodes (32) to humans (33). They are predominantly expressed in mesodermally derived tissues and genetic studies have shown that they play diverse roles during development of these tissues. Unlike other members of this family, HAND1 and HAND2 are also widely expressed in neural crest derivatives. Despite a demonstrated role for HAND2 during development of the heart (16, 17), limb (34, 35), and neural crest derivatives (36), little is known about its mode of action. In this study we have characterized the transcriptional activation domain, DNA binding site, and protein partners of HAND2 *in vitro* and *in vivo*. The extensive similarity in sequence between HAND1 and HAND2 suggested analogous functions in regulating transcription. The transcriptional activity of HAND1...
The bHLH factor Pip has a transcriptional activation domain a 40-fold higher activity than full-length NeuroD protein (3). The isolated NeuroD activation domain has been examined and HAND1 shown to function both as a transcription factor (39). The E-protein that interacts with HAND2 encoded by E12, and its disruption does not lead to defects in overall level of sequence similarity. Surprisingly, the sequence divergence that exists is localized predominantly to the basic region. Because the basic region confers DNA binding specificity, the divergent sequence would suggest that HAND1 and HAND2 dimerize with the same bHLH partners but can bind different DNA sequences. Our binding site analysis, however, suggests that the consensus binding sites for HAND2 overlaps the binding site of HAND1 (15). The ability of both HAND1 and HAND2 to dimerize with E-proteins and bind similar DNA sequences suggests that they can regulate different genes but may interact at cis-regulatory elements to regulate the expression of overlapping sets of genes.

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