The Interaction between dHAND and Arix at the Dopamine β-Hydroxylase Promoter Region Is Independent of Direct dHAND Binding to DNA*

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Dopamine β-hydroxylase (DBH) catalyzes the production of norepinephrine, and its expression defines the noradrenergic phenotype. Transcription factors dHAND, a basic helix-loop-helix protein, and Arix/Phox2a, a homeoprotein, have been demonstrated to play a role in the differentiation and maintenance of catecholaminergic neurons. Three Arix regulatory sites have been identified in the DBH promoter proximal region, but there is no such evidence for dHAND. Cotransfection with a DBH promoter-luciferase reporter construct plus dHAND or dHAND-E12 expression plasmids did not alter luciferase activity, whereas transfection with Arix resulted in a 2.5-fold stimulation of luciferase activity. However, a 5.5-fold increase was observed when Arix and dHAND were combined, and an 8-fold level of expression was observed when Arix was transfected with a dHAND mutant lacking the basic DNA-binding domain. When the homeodomain sites in the DBH promoter proximal region were mutated, all activity was lost, demonstrating dependence upon Arix-DNA interaction for transcriptional activation. In electrophoretic mobility shift assays, the addition of dHAND decreased the amount of Arix needed to elicit a mobility shift with the DBH homeodomain sites, and the dHAND basic mutant potentiated Arix binding in a manner similar to wild-type dHAND. The dHAND-Arix complex was dissociated upon the addition of an unlabeled competitor containing a homeodomain, but not upon the addition of a competitor containing E-boxes. Arix coprecipitated with antisera directed against recombinant dHAND, demonstrating direct protein-protein interactions. These results indicate that the activation of the DBH promoter by Arix is potentiated by dHAND via a mechanism independent of a direct interaction of dHAND with DNA.

Cell-specific transcription is accomplished by the precise and transient interactions of numerous and complex molecular factors. Basic helix-loop-helix (bHLH)1 proteins are transcription factors that play an important role in the development and differentiation of specific cell types such as neurons (1), appendages (2), and myocytes (3, 4). These bHLH proteins have been shown to bind to defined consensus sites, E-boxes (CANNTG), through the basic domain and require dimerization partners (via helices) to activate transcription. These proteins have been divided into several classes based on structure, function, and tissue specificity. Class I bHLH proteins (E12 and E47) are expressed ubiquitously in all tissues and can function as homodimers, but prefer to form heterodimers with other bHLH proteins (5). Class II proteins (dHAND, NeuroD, and MyoD) are expressed in specific tissues and are believed to be functionally active when complexed with a class I protein (5, 6). dHAND/HAND2 (deciduum, heart, autonomic nervous system, and neural crest cell derivatives) is a 30-kDa member of the class II bHLH protein family (7, 8) and has been detected in Xenopus, zebrafish, chicks, mice, and humans (9). dHAND is required for cardiac (7), vascular (10), and craniofacial development (11) and is an important factor in limb (12) and sympathetic neuron (13, 14) development. Recent evidence has demonstrated that the expression of genes that lead to norepinephrine production can be elicited by ectopic dHAND expression in avian embryos (15, 16).

Several of the class II bHLH proteins have been shown to associate with other transcription factors. For example, Neuromedin E47 interacts with Pitx1, a homeodomain (HD) protein, to regulate expression of the pituitary pro-opiomelanocortin gene (17), and this interaction is dependent on both transcription factors binding to the promoter. MyoD-E12 associates with myocyte enhancer factor-2A, a member of the MADS family, to activate myogenesis, but only one of these factors (either myocyte enhancer factor-2A or the MyoD-E12 complex) needs to interact with the DNA to activate transcription (3). dHAND has been shown to associate with the bHLH proteins E12 (6) and eHAND (18) and GATA4 and p300 (19); and the cardio-specific enhancer adenylsuccinate synthetase was the first target gene for dHAND (20). However, all of these studies demonstrated the dependence of dHAND binding to E-boxes within the promoter region to activate gene expression. Recently, it has been shown that ectopically expressed dHAND induces digit duplication in transgenic mice, and similar effects were observed with a dHAND mutant lacking the basic domain, but this mutant was not able to activate transcription in reporter assays when expressed with E12 or to bind to DNA in electrophoretic mobility shift assay (EMSA) (21). These latter results suggest that dHAND could activate transcription independent of direct DNA binding, but other proteins are required to recruit dHAND to the promoter region.

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The abbreviations used are: bHLH, basic helix-loop-helix; HD, homeodomain; EMSA, electrophoretic mobility shift assay; DBH, dopamine β-hydroxylase; RT, reverse transcription; HA, hemagglutinin; GST, glutathione S-transferase.

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Sympathetic neurons are derived from neural crest cells, and their development is controlled by the correct expression of several transcription factors that form a complex regulatory network rather than a linear cascade. During embryogenesis, bone morphogenetic proteins induce the expression of the bHLH protein MASH, the HD proteins NBPhox/Phox2b and Arix/Phox2a, and dHAND (1). Neural crest-derived cells treated with antisense transcript encoding dHAND genes have an 80% decrease in neurogenesis and a 60% decrease in catecholaminergic differentiation (13). Moreover, when dHAND was overexpressed in avian neural crest cells, mRNAs encoding the noradrenergic biosynthetic enzymes tyrosine hydroxylase and dopamine \( \beta \)-hydroxylase (DBH) were detected, indicating a potential role of dHAND in catecholaminergic cell development. Arix regulates norepinephrine biosynthesis by directly binding to three HD sites (ATTA) in the 5'-upstream region of the DBH gene (22–24). The DBH promoter region also contains two putative E-box motifs (CAATGG and CAAATGC), yet neither the noradrenergic phenotype are not yet known.

The purpose of this study was to investigate the interactions between dHAND and Arix at the DBH promoter region. In this study, we demonstrate that dHAND interacts directly with Arix to cooperatively activate transcription of the DBH promoter. Whereas the action of Arix is dependent upon the presence of its DNA recognition sites, the action of dHAND is independent of DNA binding.

**EXPERIMENTAL PROCEDURES**

**RT-PCR**—RNA was isolated from tissues and cells using TRIzol (Invitrogen) according to the manufacturer’s directions. RNA (2.5 \( \mu \)g), oligo(dT) (500 ng; Invitrogen), and dNTPs (10 mM each) were incubated at 70 °C for 10 min and transferred to ice for 2 min. Dithiothreitol (10 mM) and 5 \( \times \) buffer were added and incubated at 42 °C for 2 min. Superscript reverse transcriptase (200 units; Invitrogen) was added and incubated at 42 °C for 50 min. The reaction was stopped by incubating the samples at 70 °C for 10 min. 2 \( \mu \)l of the RT reaction was used for PCR. The PCR mixture contained 100 ng of each primer, 1.5 mM MgCl\(_2\), and 200 ng dNTP. Mixtures were heated at 94 °C for 10 min and transferred to ice for 1 min for 30 cycles, followed by a 10-min polymerization step at 72 °C. Samples were resolved by electrophoresis on 3% low melting point agarose gels. Primers used for amplification of cDNAs are as follows: mouse and rat dHAND, 5'-TCCAGATTCAAGACTGTGG-3' and 5'-CGTTGCTGCCTAGTGTGC-3'; human hand, 5'-GGGAGATGAGCAAGACG-3' and 5'-CGTTGCTGCCTAGTGTGC-3'; mouse, rat, and human tyrosine hydroxylase, 5'-CATTGGACTTGCATCTCTGG-3' and 5'-GTTCGAGCCTGTCTGGG-3'; mouse, rat, and human Arix, 5'-GGAAACCTTTCTTTGGTTC-3' and 5'-ACCAAGCTGCTCCATCGCT-3'; mouse and rat DBH, 5'-AAGGTTGTTAGTCTGCTGC-3' and 5'-CACACATCTCCGAGAAGCTTC-3'; human DBH, 5'-TCCAGACAGTGACTTGCAG-3' and 5'-CGTTGCTGCCTAGTGTGC-3'; mouse and rat glyceraldehyde-3-phosphate dehydrogenase, 5'-CAGGACACCTTCTCCAGG-3' and 5'-GGACTGCTGCTCCTAGTGTGC-3'; and mouse and rat \( \beta \)-actin, 5'-GGTCTCTCTTCCTCTTGTGC-3'.

**In Vitro Protein Interactions**—For the in vitro protein-protein interaction assay, dHAND, dHAND-\( \beta \)-basic, and c-Jun were transcribed in \( \beta \)-galactosidase-driven expression vectors and Arix was a generous gift from Dr. Marthe Howard (Medical College of Ohio). To clone dHAND into the mammalian expression vector pcDNA3.0, a Bam/H\_Xhol fragment from dHAND-BSKs containing the complete coding sequence was inserted into the vector. To construct dHAND-HA6.1, a full-length dHAND PBMXH\_Xhol fragment was ligated to an oligonucleotide to create a Bam/H\_Xhol site, and then the end of the Xhol site of HA6.1. To create His-dHAND, the XhoI fragment from dHAND-HA6.1 was inserted into pET28b (Novagen). The construction of the DBH-\( \beta \)-galactosidase reporter plasmid containing the promoter and the 5'-flanking sequence was described previously (20). dHAND-BSKs was a generous gift from Dr. Mathew Thayer (Oregon Health and Sciences University). pcDNA3.1-dHAND-wt (where "wt" is wild type) pcDNA3.1-dHAND-R108–111EDE (dHAND-EDE), and pcDNA3.1-dHAND-basic deletion (dHAND-\( \beta \)-basic) were generously provided by Dr. Eric Olson (University of Texas). To create His-tagged dHAND-\( \beta \)-basic, the StuI/\_PstI fragment of pcDNA3.1-dHAND-\( \beta \)-basic was cloned into pET28b-His-dHAND. All dHAND clones were confirmed by DNA sequencing.

**Transfections and Reporter Assays**—NT2 teratocarcinoma cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 10 \( \mu \)g/ml streptomycin, and 10 \( \mu \)g/ml penicillin. Cells were plated at a density of 1.0 \( \times \)10\(^6\) cells/well 1 day prior to transfection. All transfections were performed in 6-well plates with GeneFecter (Venn Nova, Inc.) according to the manufacturer’s recommendations with 0.2 \( \mu \)g of pRL-null (Promega), 0.1 \( \mu \)g of DBH-luciferase, 0.4 \( \mu \)g of Arix, 0.4 \( \mu \)g of E12, 0.5 \( \mu \)g of dHAND-HA, 0.8 \( \mu \)g of wild-type dHAND, 0.8 \( \mu \)g of dHAND-EDE, and 0.8 \( \mu \)g of dHAND-\( \beta \)-basic. Initial studies determined the amount of each dHAND construct that produced maximal luciferase activity. The total amount of DNA in each transfection was adjusted to 2.8 \( \mu \)g using the backbone vector pcDNA3.0. Experiments were done in triplicate and repeated three times. All cells were transiently transfected with pRL-null as a control for transfection efficiency.

**Expression of Recombinant Proteins**—The pET and pGEX plasmids were transformed into the bacterial host BL21(DE3). Log-phase bacterial cultures were treated with 1 mM isopropyl-\( \beta \)-thiogalactopyranoside and incubated at an initial 4 °C for 4 h at 37 °C. Bacteria were pelleted, resuspended in sonication buffer (23), and sonicated. Soluble fractions were applied to nickel-nitrilotriacetic acid-agarose (QIAGEN Inc.), and His-tagged proteins were eluted by stepwise gradients of imidazole-containing elution buffer. The concentration of His-tagged proteins was estimated by Western analysis using nickel-labeled horse-radish peroxidase (Kirkgaard & Perry Laboratories, Inc.) The GST-tagged MyoD soluble fraction was applied to glutathione-agarose (Sigma) and eluted with 20 mM glutathione. Protein concentration was determined by the Bradford assay (Bio-Rad).

**EMSA**—To create a probe spanning rat DBH sequence 183 to 57, the primer pair 5'-ATGATGTCAAGCTTTAGCTGTTGACTG-3' and 5'-CAAATGTGATTAGGTAC-3' corresponding to bases 150 to 183 of the sequence (22) and 5'-CAATGTGATTAGGTAC-3' (corresponding to bases 73 to 57 of the mouse DBH promoter) was \( \beta \)-32P-end-labeled using polynucleotide kinase and used to amplify the DBH DNA. The PCR was the same as described above with 100 ng of template. The resulting DNA fragments were resolved by electrophoresis on 3% low melting point agarose gel. The correct band was excised and digested by HindIII/PstI and electrophoresed, resuspended in sonication buffer (23), and sonicated. Soluble fractions were applied to nickel-nitrilotriacetic acid-agarose (QIAGEN Inc.), and His-tagged proteins were eluted by stepwise gradients of imidazole-containing elution buffer. The concentration of His-tagged proteins was estimated by Western analysis using nickel-labeled horse-radish peroxidase (Kirkgaard & Perry Laboratories, Inc.) The GST-tagged MyoD soluble fraction was applied to glutathione-agarose (Sigma) and eluted with 20 mM glutathione. Protein concentration was determined by the Bradford assay (Bio-Rad).

**Co-immunoprecipitation of Arix**—SK-N-SH cells were transfected with 10 \( \mu \)g of dHAND-HA in pcDNA3; and after 2 days, 300 \( \mu \)g/ml G418 (Invitrogen) was added. After 3 weeks, cells were isolated and maintained with G418. The expression of dHAND-HA was confirmed by Western blotting. Cells were lysed with radiolabeled immunoprecipitation assay buffer, and cell samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline/Tween and 5% milk for 1 h at room temperature, incubated overnight at 4 °C with rat anti-HA antibody (200 ng/ml in Tris-buffered saline/Tween and 5% milk; Roche Applied Science), washed, incubated with sheep anti-rat horseradish peroxidase antibody (1:6000 in Tris-buffered saline/Tween and 5% milk) and then exposed on film with Super Signal West Pico chemiluminescence solution (Pierce). Nuclear proteins were extracted from SK-N-SH cells and SK-N-SH cells that had been stably transfected with dHAND-HA using the Celllytic NuClear extraction kit (Sigma) and dialyzed against 20 mM HEPES, 100 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, and 50 mM Tris. Nuclear proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose.
mM KCl, 20% glycerol, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol to remove salts. Nuclear proteins (300 μg) were incubated with 200 ng/ml anti-HA antibody overnight and precipitated with 30 μl of protein G-agarose (Roche Applied Science). Precipitates were washed five times with 10 mM Tris, 200 mM NaCl, 1 mg/ml bovine serum albumin, 0.05% Nonidet P-40, 10 mM NaF, and 1 mM phenylmethylsulfonyl fluoride; boiled, subjected to SDS-PAGE analysis; and transferred to membranes. After the blocking step, membranes were incubated overnight at 4 °C with chicken anti-Arix antibody (1:500), washed, incubated with goat anti-chicken horseradish peroxidase antibody (1:5000), and washed; and immunoreactive bands were visualized with a 50:50 mixture of Super Signal West Pico and Femto chemiluminescence solutions.

RESULTS

Distribution of dHAND mRNA—To evaluate the distribution of dHAND in catecholaminergic cells, the expression of dHAND mRNA in various cell types and tissues was evaluated by RT-PCR. dHAND mRNA was not detected in NT2 teratocarcinoma cells, but was detected in adrenal gland-derived PC-12 cells and in the following neuroblastoma cell lines: CATH.a, M17, SH-SY5Y, and SK-N-SH (Fig. 1 and Table I). dHAND mRNA (adult rat and mouse) was not detected in mouse kidney or pons medulla, but was detected in mouse adrenal gland, rat heart, and sympathetic ganglia. The expression of dHAND mRNA in sympathoadrenal tissue correlated with the expression of tyrosine hydroxylase, DBH, and Arix. Previous work has demonstrated the expression of dHAND in fetal and embryonic human sympathetic neuronal tissue (14), but this is the first evidence of dHAND mRNA in adult sympathoadrenal tissue. All RT reactions were run with and without Superscript, and no products were observed in the treatments lacking Superscript (data not shown).

Transient Transfection Assays—The DBH promoter contains three HD sites (ATTA) that interact with Arix (22, 23, 24) and two putative E-boxes (CANNTG), which could be potential dHAND-binding sites (Fig. 2). To investigate the ability of dHAND to interact with Arix at the DBH promoter region, NT2 cells were transiently transfected with the DBH(-232/+10)-luciferase reporter plasmid plus various expression plasmids. When cells were transfected with the pcDNA3.0 backbone plasmid, basal levels of expression were observed (Fig. 3A). Transfection of NT2 cells with dHAND, dHAND-E12, or MyoD-E12

![Distribution of dHAND RNA transcripts.](image)

![Details of the sequence and structure of the rat DBH promoter region (−209 to +10).](image)

| Cell type/tissue | dHAND | TH | DBH | Arix |
|-----------------|-------|----|-----|-----|
| NT2             | –     | +  | +   | +   |
| PC-12           | +     | +  | +   | +   |
| CATH.a          | +     | +  | +   | +   |
| M17             | +     | +  | +   | +   |
| SH-SY5Y         | +     | +  | +   | +   |
| SK-N-SH         | +     | +  | +   | +   |
| Mouse kidney    | –     | –  | –   | –   |
| Mouse pons medulla | – | –  | –   | –   |
| Mouse adrenal gland | – | –  | –   | –   |
| Rat heart       | –     | –  | –   | –   |
| Rat sympathetic ganglia | – | –  | –   | –   |

*Tyrosine hydroxylase.*
expression plasmids resulted in luciferase activity that was unchanged from basal activity. When the Arx plasmid was added to the transfection, the relative luciferase activity increased to 2.5-fold over basal levels. This amount of Arx is below that which maximally stimulates the DBH-luciferase constructs (23). When dHAND was added in the presence of Arx, the activity increased to 5.3-fold; however, when similar amounts of MyoD were added with Arx, expression was similar to that with Arx alone. Therefore, it appears that, together, dHAND and Arx interact synergistically to activate the DBH promoter.

Many bHLH proteins have been shown to bind to consensus E-boxes via the basic domain (5). However, recent data have indicated that dHAND may be able to mediate expression via protein-protein interactions independent of DNA binding (21, 26). To determine whether the basic DNA-binding domain of dHAND is required for the activation of the DBH promoter, NT2 cells were transfected with two different dHAND mutants. dHAND-EDE has a mutation in the basic region (R109E/R110D/R111E), and dHAND-Δbasic lacks the entire 10-amino acid basic region. Previous work has demonstrated that these mutants cannot activate transcription from a consensus E-box.

**Fig. 3. Transcriptional activities of dHAND and Arx on the DBH promoter.** A. NT2 cells were transfected with the DBH(-232/+10)-luciferase reporter and pcDNA3.0 expression vectors encoding dHAND, E12, Arx, and MyoD. B. NT2 cells were transfected with the DBH-luciferase reporter and pcDNA3.0 expression vectors encoding Arx and wild-type and mutant dHAND (dH) proteins (see “Experimental Procedures” for descriptions). C. NT2 cells were transfected with wild-type and mutant DBH-luciferase reporters and pcDNA3.0 expression vectors encoding Arx and dHAND. Values are expressed as -fold activation of the reporter gene relative to the level of the expression reporter alone and represent means ± S.E. of three transfections.
in a transient transfection assay, but they are still able to cause limb abnormalities similar to those caused by wild-type dHAND in transgenic embryos (21). No activation was observed when NT2 cells were transfected with dHAND, dHAND-EDE, or dHAND-basic alone (Fig. 3B), and no activation was observed when E12 was added to the assay (data not shown). As observed in the previous experiments, transfection with Arix alone resulted in a 2.3-fold increase in expression, whereas Arix and dHAND caused a 5.2-fold increase in expression (Fig. 3B). When dHAND-EDE was transfected with Arix, a 6.6-fold increase in expression was observed; and when dHAND-basic was transfected with Arix, an 8.1-fold increase was observed (Fig. 3B). Because expression of the DBH promoter was observed with Arix and with both of the dHAND basic mutants, it appears that the basic region of dHAND is not needed for activation of the DBH promoter, suggesting that
activation by dHAND is independent of DNA binding.

Previous research has demonstrated that Arix activates transcription of the DBH promoter via three HD sites and that transcriptional activity is lost if these sites are mutated (25). To determine whether dHAND and Arix contact the DBH promoter through the HD sites, NT2 cells were transfected with DBH-luciferase plasmids containing mutations in the HD sites. Transfection of wild-type DBH-luciferase with dHAND resulted in basal levels; transfection with Arix resulted in a 1.8-fold increase in luciferase activity; and the addition of dHAND to Arix doubled the luciferase activity elicited with Arix alone (Fig. 3C). In DBH-luciferase constructs containing a mutation in HD1 plus HD2 or HD3, basal activity was observed in cells treated with Arix, but 2.2- and 1.7-fold increases in activity were observed when dHAND was added to Arix. These results demonstrate that dHAND can stimulate the transcriptional activation by Arix though either HD site. Without the three HD sites, no activity was observed when cells were transfected with pcDNA3, dHAND, Arix, or dHAND plus Arix. Moreover, no activity was observed when cells were cotransfected with the DBH-luciferase construct containing mutations in all three HD sites and dHAND-EDE plus Arix or dHAND basic plus Arix (data not shown). These data indicate that each of the HD sites contributes to the activation of the DBH promoter region by Arix and dHAND and that the transcriptional activation is dependent upon the presence of the Arix-binding sites.

Interactions between dHAND, Arix, and the DBH Promoter in Vitro—To further characterize the interaction between Arix and dHAND, we utilized EMSA to investigate the physical interactions between dHAND and Arix at the DBH promoter proximal region. The DBH (−183/−57) probe containing all three HD sites and both putative E-box motifs (Fig. 2) was used to assay DNA-protein interactions. When His-dHAND was incubated with the radiolabeled DBH probe, there was no difference between it and the control lane of the probe alone (Fig. 4A, lane 1 and 3). Moreover, when dHAND was incubated with His-E12, no shift was observed (data not shown). When a non-saturating concentration of His-Arix was incubated with the DBH promoter, several bands were observed (Fig. 4A, lane 5). We believe these bands to be dimer and multimer complexes of Arix binding to single and multiple HD sites on the DBH probe. When anti-Arix antibody was added, the bands were shifted to a large complex that could not be resolved by electrophoresis (Fig. 4A, lane 6). When four times as much Arix was incubated with the DBH probe, a high molecular mass band was observed, presumably the DNA-protein complex containing saturating amounts of Arix, and this single band was shifted upon the addition of anti-Arix antibody (Fig. 4A, lanes 9 and 10). Anti-Arix antibody had no effect on the probe alone or on His-tagged dHAND (Fig. 4A, lanes 2 and 4). When a non-saturating concentration of Arix was incubated together with dHAND, slower mobility complexes were observed, and these bands were shifted upon the addition of anti-Arix antibody (Fig. 4A, lanes 7 and 8). No bands were observed when GST-MyoD was incubated with the DBH promoter, and the observed bands were similar to those with Arix alone when GST-MyoD was incubated with Arix (Fig. 4A, lanes 11 and 12). These results indicate that dHAND and Arix interact at the DBH promoter proximal region and that dHAND facilitates the binding of Arix to the DNA. This interaction appears to be specific to dHAND and Arix because the slow mobility complex was not observed when Arix was incubated with the bHLH protein MyoD.

To investigate the interactions between dHAND and Arix with the individual HD sites, EMSAs were carried out with smaller oligonucleotides containing HD3 or both HD1 and HD2 (Fig. 4B). When Arix alone was incubated with the ATTA probe containing HD3 and E-box-2, a faint band was observed (Fig. 4B, lane 2). As before, dHAND did not bind to the probe (Fig. 4B, lane 3). However, when the same amounts of Arix and dHAND were incubated together, a strong band appeared (Fig. 4B, lane 4). Similar results were observed using the DB1 probe, which contains two HD sites separated by 6 bases. When Arix or dHAND alone was incubated with the DB1 probe, no binding was observed (Fig. 4B, lanes 6 and 7), but the combination of Arix and dHAND resulted in the formation of strong DNA-protein complexes (lane 8). The DB1 probe does not contain an E-box, which further supports the evidence that dHAND and Arix contact the DBH promoter proximal region through the HD sites and not through the E-boxes.

EMSA analysis further corroborated the evidence that the basic DNA-binding region is not needed for dHAND to functionally interact with Arix at the DBH promoter region. Previous work has demonstrated that the construct lacking the entire basic region is not able to bind to DNA in gel shift assays when incubated with E12, but is able to activate expression of limb formation in transgenic mice (21). Alone, neither His-dHAND nor His-dHAND basic to the −187/−57 DBH probe (Fig. 4C, lanes 3 and 4), but when Arix was included, a high molecular mass band was observed (lanes 5 and 6). Thus, the interactions between dHAND and Arix at the DBH promoter region are not dependent on dHAND binding to the DNA.

To determine where the dHAND-Arix complex binds to the DBH promoter region, EMSA analysis was conducted with unlabeled competitors containing either the two DBH E-box motifs or HD3 (Fig. 4D). When Arix and dHAND were incubated with an unlabeled competitor containing both E-box motifs from the DBH promoter, the observed shift was similar to that obtained with Arix and dHAND without competitor oligonucleotides (Fig. 4D, lanes 4 and 5). However, when Arix and dHAND were incubated with an unlabeled competitor containing HD3, the high molecular mass band was not observed (Fig. 4D, lane 6). Therefore, it appears that the dHAND-Arix com-
plex binds to the DBH promoter via HD sites and not through the E-boxes.

To quantify the ability of dHAND to enhance the binding specificity of Arix for the DBH promoter, increasing amounts of Arix were incubated alone or with dHAND or MyoD. When Arix was incubated with the DBH probe, 2 μg Arix was needed to shift 50% of the probe (Fig. 5). The sigmoidal shape of the curve indicates a positive cooperativity of Arix for the DBH sites, and the curve has a cooperativity index ($[S]_{0.9}/[S]_{0.1}$) of 1.96. Adachi et al. (23) also demonstrated that Arix binds with moderate cooperativity to the DB1 probe, which contains HD1 and HD2. When Arix was incubated with MyoD, the shape of the curve was similar to that obtained with Arix alone, and the cooperativity index was comparable (1.80). However, when dHAND was incubated with Arix, only 1 μg/μl Arix was needed to shift 50% of the probe, and this sigmoidal curve has a higher cooperativity index of 3.13. These results demonstrate that dHAND can specifically potentiate the binding of Arix to its recognition sites on the DBH promoter.

**Protein-Protein Interactions between dHAND and Arix**—To determine whether dHAND and Arix directly contact each other, glutathione-agarose beads containing the GST-Arix fusion protein were incubated with in vitro translated 35S-labeled dHAND, dHAND-Δbasic, or c-Jun. c-Jun is an AP1 protein that binds the DBH AP1/cAMP response element, but does not make any direct contacts with Arix (27). dHAND did not interact with the glutathione-agarose beads, but when GST-Arix-bound beads were added, a strong signal was observed (Fig. 6A), demonstrating interaction between Arix and dHAND. Moreover, direct contacts were observed when the dHAND-Δbasic mutant was incubated with GST-Arix-bound beads. However, when GST-Arix was incubated with c-Jun, there was no significant difference in the background binding of c-Jun to either the beads alone or GST-Arix-bound beads.

Co-immunoprecipitation assays further confirmed the interaction between Arix and dHAND. Nuclear extracts from SK-N-SH cells alone or SK-N-SH cells that had been stably transfected with dHAND-HA were immunoprecipitated with anti-HA antibody and then subjected to Western analysis with anti-Arix antibody. No signal was observed in the SK-N-SH cells immunoprecipitated with either anti-HA antibody or protein G-agarose (Fig. 6B); however, in the stably transfected cells, Arix was precipitated with anti-HA antibody, but not with protein G-agarose. These results confirm the hypothesis that dHAND and Arix interact directly within the cell.

**DISCUSSION**

Tissue-specific gene expression encompasses the integration of multiple influences, such as extracellular signals and intracellular transcription factors, to achieve specialized cell function. In this work, we have investigated the interactions between two noradrenergic transcription factors, dHAND and Arix, at the DBH promoter proximal region. Previous work has shown the importance of dHAND in the development and maintenance of the noradrenergic phenotype (13, 15, 16); but until now, there has been no evidence that dHAND directly interacts with the noradrenergic biosynthetic gene regulatory regions. In this study, we have demonstrated that dHAND and Arix cooperate to activate transcription of the DBH promoter and that the dHAND-Arix complex contacts the DNA through the HD sites. Moreover, we have shown that the basic DNA-binding domain of dHAND is not required for its potentiation of Arix function. These results establish a new function for dHAND as a transcriptional coactivator.

Homeobox (ATTA) sequences occur 11,183,064 times in the human genome; and as a result, HD proteins must interact with other proteins to achieve tissue-specific regulation. Many HD proteins have been shown to bind to other HD proteins, e.g.
dHAND Interacts Independent of DNA Binding

Ead and Ubx, and to raise DNA-binding specificity and thereby alter target gene selection. By themselves, Ubx binding is weak, and Ead is undetectable in EMSA; however, the DNA-binding affinity of both proteins increases when incubated together (28, 29). Our results indicate that Arix and dHAND can act as transcription partners to activate DBH gene expression. Transfections with Arix and dHAND had 2-fold higher activity than those with Arix alone (Fig. 3A), and EMSA analysis further demonstrated a positive binding cooperation between Arix and dHAND at the DBH promoter region (Figs. 4A and 5).

Furthermore, dHAND was shown to associate with Arix in both in vitro and in vivo precipitation assays (Fig. 6). In vivo, this interaction between Arix and dHAND could enhance the selectivity of Arix for the appropriate HD sites. In developing autonomic neurons, only cells containing both dHAND and Phox2 (Arix) proteins express noradrenergic traits (16), and this tripartite Arix-dHAND-DNA interaction could be the basis for the noradrenergic phenotype.

Previous work has demonstrated that Arix can activate the DBH promoter by binding to three HD sites in the promoter proximal region (22, 23, 24). The DBH promoter also contains two putative E-boxes, and it was originally thought that dHAND could activate transcription by binding to these sites. However, dHAND did not bind to the potential E-boxes of the DBH gene (data not shown), and the E-box competitor oligonucleotides did not compete for complex formation on the DBH DNA probes, whereas the HD oligonucleotides were effective competitors (Fig. 4D). These data demonstrate that the Arix-dHAND DNA-protein interaction is mediated through the HD sites and not through the E-boxes.

Traditionally, bHLH proteins have been shown to activate transcription by binding to E-boxes in the promoter region of target genes (5). It has recently been demonstrated that dHAND can elicit a distinct phenotype in a DNA binding-independent manner, which suggests that the DNA-binding domain of dHAND might function in a more complicated manner than the traditional models of bHLH proteins. Misexpression of a dHAND-Δbasic construct in mice induces ectopic digits, as does intact dHAND, even though dHAND-Δbasic is not active in cell transfection assays, suggesting that other proteins are necessary to bring dHAND to the promoter region (21). Other researchers have shown that dHAND-Δbasic-E12 can activate the atrial natriuretic peptide promoter in a manner similar to wild-type dHAND-E12 (2.7-fold versus 3.1-fold activation), and these values are similar to those of a promoter lacking E-boxes (2.4-fold versus 2.8-fold) (26). However, these values are low in comparison with the 22-fold stimulation by the combination of dHAND, E12 and Nkx2.5, and the interactions between dHAND-Δbasic, E12, and Nkx2.5 were not reported. In the study reported herein, dHAND-Δbasic plus Arix transactivated the DBH promoter to a greater extent compared with intact dHAND plus Arix (Fig. 3B). One possible reason for this observation is that without the basic region, a greater portion of dHAND could be bound to Arix and not to other proteins or regions on the host or plasmid DNA. When combined with Arix, the dHAND-Δbasic mutant was as effective as wild-type dHAND in potentiating the interaction of Arix with DNA (Fig. 4C) and could associate with Arix in protein-protein interactions in vitro (Fig. 6A). These results indicate that the basic DNA-binding region of dHAND is not required for association with Arix and the DBH promoter region. Further work will determine the specific regions of interaction between Arix and dHAND.

dHAND could be functioning in several ways with Arix at the DBH promoter region. Because dHAND could successfully precipitate Arix (Fig. 6B), we believe that dHAND and Arix directly interact at the DBH promoter region to cooperatively activate transcription. Recently, dHAND has been shown to make direct contacts with the HD protein Nkx2.5 (26) and GATA4 and p500 (19); and together, these factors are able to activate transcription of the atrial natriuretic peptide promoter. However, the interaction between GATA4 and dHAND does not alter either the magnitude or the pattern of DNA binding of either protein (19). The conclusions from that study suggest that contact with the coactivator p500 is needed for the cooperative interaction between dHAND and GATA4. Because protein-protein interactions between Arix and dHAND were observed in the absence of DNA (Fig. 6A), dHAND could bind to Arix, inducing conformational changes prior to association with the HD sites. Additionally, dHAND could prevent the dissociation of Arix from the HD sites, thereby stabilizing the interaction between Arix and the DBH promoter. Raising the amount of Arix in the EMSA produced results similar to those obtained with Arix plus dHAND (Fig. 5), which supports the latter hypothesis. Alternatively, dHAND could be acting as an enzyme to modify Arix. Previous work has demonstrated that dephosphorylated Arix gains stronger DNA-binding activity and stabilizes the transcriptional machinery (30); however, there has been no evidence to date of any bHLH protein acting as a phosphatase. Further work is needed to determine which regions are important for the protein-protein interactions and if any structural alterations are important for cooperative DNA binding.

Hong et al. (31) have shown that a promoterless luciferase reporter gene can be activated by bHLH proteins such as dHAND and eHAND. However, we never observed any activity when cells were transfected with any of the bHLH proteins alone (Fig. 3A). Other researchers have also found that transfections with dHAND alone cannot significantly activate transcription of luciferase promoters (6, 19). Therefore, it does not appear that dHAND can activate the transcription of the luciferase gene or the flanking sequences.

Gestblom et al. (14) reported the presence of dHAND RNA in several neuroblastomas, neuroblastoma cell lines, and fetal/embryonic tissues. Our results agree with and further expand the distribution of dHAND in neuroblastoma cell lines. We detected dHAND RNA in adult mouse adrenal glands, but Gestblom et al. (14) did not detect dHAND RNA in postnatal human adrenal glands. However, they conceded that definitive conclusions could not have been drawn due to the limited amount of tissue. Additionally, there could be differences in dHAND expression between adult mice and humans. Ectopic expression of dHAND in ciliary ganglia has been shown to preserve noradrenergic gene expression (16), and we observed a faint signal in adult rat sympathetic ganglia, further supporting the hypothesis that dHAND is important for the maintenance as well as the development of the sympathetic phenotype. dHAND RNA was not detected in the pons medulla, which contains the locus ceruleus, and dHAND is not believed to be involved in the development of this class of neurons (1). The expression pattern of dHAND RNA in the sympathetic neural lineage follows the expression of other noradrenergic markers such as Arix, tyrosine hydroxylase, and DBH. Additional research is needed to determine whether dHAND, Arix, and Phox2b are involved in the regulation of tyrosine hydroxylase gene expression in addition to DBH.

Here, we have demonstrated that dHAND and Arix can synergistically activate the DBH promoter and that dHAND potentiates the binding of Arix to the DBH promoter. Moreover, we have shown that dHAND acts in a DNA binding-independent fashion with Arix to activate transcription. The hypothesis...
that dHAND and Arix interact to elicit norepinephrine biosynthesis has been suggested in developmental studies (16); but until now, there was no direct evidence obtained using an endogenous promoter. Finally, this evidence also indicates that dHAND and Arix not only are critical for the development of the noradrenergic phenotype, but also act directly in its maintenance. Taken together, these results demonstrate that dHAND is a coactivator of DBH-dependent gene expression, which appears to be mediated by direct protein-protein interactions between dHAND and Arix.

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Note Added in Proof—While this manuscript was in review, Xu et al. (Xu, H., Firulli, A. B., Zhang, X., and Howard, M. J. (2003) Dev. Biol. 262, 183–193) reported similar transcriptional activation between dHAND and Arix with DBH promoter. However, they did not observe an interaction between in vitro translated dHAND and Arix.

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The Interaction between dHAND and Arix at the Dopamine β-Hydroxylase Promoter Region Is Independent of Direct dHAND Binding to DNA

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