The Transcription Factors GATA4 and dHAND Physically Interact to Synergistically Activate Cardiac Gene Expression through a p300-dependent Mechanism*

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An intricate array of heterogeneous transcription factors participate in programming tissue-specific gene expression through combinatorial interactions that are unique to a given cell-type. The zinc finger-containing transcription factor GATA4, which is widely expressed in mesodermal and endodermal derived tissues, is thought to regulate cardiac myocyte-specific gene expression through combinatorial interactions with other semi-restricted transcription factors such as myocyte enhancer factor 2, nuclear factor of activated T-cells, serum response factor, and Nkx2.5. Here we determined that GATA4 also interacts with the cardiac-expressed basic helix-loop-helix transcription factor dHAND (also known as HAND2). GATA4 and dHAND synergistically activated expression of cardiac-specific promoters from the atrial natriuretic factor gene, the b-type natriuretic peptide gene, and the o-mysin heavy chain gene. Using artificial reporter constructs this functional synergy was shown to be GATA site-dependent, but E-box site-independent. A mechanism for the transcriptional synergy was suggested by the observation that the bHLH domain of dHAND physically interacted with the C-terminal zinc finger domain of GATA4 forming a higher order complex. This transcriptional synergy observed between GATA4 and dHAND was associated with p300 recruitment, but not with alterations in DNA binding activity of either factor. Moreover, the bHLH domain of dHAND directly interacted with the CH3 domain of p300 suggesting the existence of a higher order complex between GATA4, dHAND, and p300. Taken together with previous observations, these results suggest the existence of an enhanceosome complex comprised of p300 and multiple semi-restricted transcription factors that together specify tissue-specific gene expression in the heart.

The zinc finger-containing transcription factor GATA4 is expressed in multiple organs derived from both endodermal and mesodermal origins, where it regulates tissue-specific gene expression through interactions with other semi-restricted transcription factors. In cardiac myocytes, GATA4 is thought to play a particularly important role in regulating expression of most cardiac-expressed genes, including o-mysin heavy chain (o-MHC),1 cardiac troponin-C, atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), cardiac troponin-I, sodium/calcium exchanger, cardiac-restricted ankyrin repeat protein, A1 adenosine receptor, m2 muscarinic receptor, and myosin light chain 1/3 (1–13). In addition to directly controlling cardiac structural and regulatory gene expression, cardiac-expressed GATA factors indirectly support tissue-specific gene expression by regulating expression of other transcription factors. For example, GATA factors regulate developmental expression of the homeodomain-containing transcription factor Nkx2.5, myocyte enhancer factor-2 (MEF2), and dHAND in the heart by providing a reinforcing transcriptional regulatory circuit mediated through direct promoter interactions (14–17). GATA4 was shown to directly interact with Nkx2.5 through the C-terminal zinc finger domain and the helix III region of the homeodomain present within each factor, respectively (18–20). GATA-4 also physically interacts by way of its C-terminal zinc finger with nuclear factor of activated T-cells (NFAT) and MEF2 (21, 22). Finally, GATA4 directly interacts with the MADS box-containing transcription factor serum response factor (SRF), which together synergistically regulate expression of the ANF and o-actin genes in cardiomyocytes (23, 24).

The studies discussed above suggest that cardiac-expressed GATA factors interact with an array of heterotypic transcription factors in the heart. In addition to transcription factor interactions, GATA4 interacts with discrete transcriptional co-activators or general repressors. For example, GATA4 was recently shown to directly interact with p300/CBP (cAMP-response element-binding protein-binding protein) resulting in synergistic gene activation (25). The N- and C-terminal zinc finger domains of GATA4 directly interacted with the cysteine/histidine-rich (CH3) region of p300 (25). Given the ability of p300/CBP to interact with a heterogeneous array of transcription factors (reviewed in Ref. 26), the observed GATA4-p300 interaction suggested a mechanism whereby a diverse array of

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1 The abbreviations used are: o-MHC, o-mysin heavy chain; ANF, atrial natriuretic factor; BNP, b-type natriuretic peptide; MEF2, myocyte enhancer factor-2; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; bHLH, basic-helix-loop-helix; CBP, cAMP-response element-binding protein-binding protein; CMV, cytomegalovirus; NFAT, nuclear factor of activated T-cells; SRF, serum response factor.
cardiac-expressed transcription factors could simultaneously interact through a p300 scaffold. GATA4 also interacts with the transcriptional modifying protein friend of GATA-2 (FOG-2) through a physical interaction involving the N-terminal zinc finger of GATA-4 (27–29). This interaction is conserved in Drosophila where the friend of GATA-2 homologue, U-shaped (Ush), interacts with panner, a GATA homologue (30). Interestingly, GATA4, FOG-2, and p300 gene-targeted mice each die during embryogenesis with significant cardiac abnormalities (31–35).

Like GATA4 gene-targeted mice, disruption of the gene encoding the transcription factor dHAND results in embryonic lethality because of cardiac abnormalities, suggesting non-redundant roles for each of these factors in specifying developmental gene expression in vitro (36). Whereas less is understood of the manner in which dHAND regulates target genes in the heart, PCR-mediated site selection identified a series of specific E-box consensus elements verifying the ability of dHAND to bind DNA (37). Here we demonstrate that the transcription factors GATA4 and dHAND physically interact with one another to synergistically regulate expression of cardiac gene promoters. The identified functional interaction is mediated through GATA, but not E-box DNA-binding sites, suggesting a dHAND-binding site-independent mechanism of regulation. Finally, dHAND was shown to physically interact with the transcriptional co-activator p300, which was necessary for functional synergy with GATA4. These data suggest a paradigm whereby cardiac-expressed transcription factors form large multisubunit complexes in conjunction with p300/CBP.

### Materials and Methods

**Plasmid Constructs**—GATA4-ΔN (N-terminal zinc finger) in the pMT2 expression vector was generated using PCR to delete amino acids 216–240, whereas the ΔC construct deleted amino acids 270–294 of GATA4. Expression vectors encoding GATA4 amino acids 253–441, wild type and site-specific mutants, were generated by PCR and subsequently subcloned into the pcDNA3.1-His vector (25). pcDNA3.1-pFlag-GATA4 was described previously (25). pFLAG-CMV-2BAP encoding a FLAG-tagged bacterial alkaline phosphatase (Sigma) was used as a control plasmid in mammalian transfection experiments. The GATA4 C-terminal zinc finger site-specific mutant expression plasmids were described previously (25), as was pcDNA-His-dHAND (37). pcMV-bHLH-Nuc-Myc is a mammalian expression vector encoding the bHLH region of dHAND (amino acids 98–157), which was cloned as a NcoI-Xhol fragment in-frame with three nuclear localization signals contained within the pcMV/NuMyc vector (Invitrogen). For construction of full-length dHAND ΔC construct, 157 amino acids were deleted using a combined PCR and ligase chain reaction technique as described previously (25). The dHAND E box artificial reporter 4xElA-TATA-Luc was generated by cloning 4 copies of the optimized dHAND-binding site (CATCTG) into pTATA-Luc at a XhoI site (37). The pTATA-Luc contains the TATA box derived from the α-MHC minimal promoter in pGL2 basic vector (Promega) (25).

**GST pull-down assays**—HELa cells were transfected with pFlag-GATA4 and CMV-ΔH-bHLH-Nuc-Myc. The cells were lysed at 4°C in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). Lysates were cleared by centrifugation at 18,000 × g for 10 min. Lysate proteins were immunoprecipitated overnight at 4°C with FLAG antibody-agarose (Sigma). The agarose was washed and the bound proteins were resolved in SDS-PAGE and Western blotted. The blot was incubated with mouse anti-Myc (Sigma). A T7 mouse monoclonal antibody was purchased from Novagen, whereas GATA4 anti-serum was purchased from Santa Cruz.

**GST Pull-down assays**—All GST fusion proteins were overexpressed in Escherichia coli BL21 cells. Binding assays were performed with labeled proteins synthesized in vitro using the TNT coupled reticulocyte lysate system (Promega) in the presence of 35S-labeled methionine (Amersham Biosciences) as described previously (25). Equal amounts of immobilized GST fusion proteins were incubated for 2 h at 4°C with 10 μl of 35S-labeled proteins in GST binding buffer containing 40 mM Hepes, pH 7.2, 50 mM Na acetate, pH 7.0, 200 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, protease inhibitors, and 2 μg of bovine serum albumin/ml. After four washes in GST binding buffer, beads were boiled in SDS sample buffer to elute bound protein, which was subsequently resolved by SDS-PAGE and analyzed by autoradiography.

**Transient Transfection Assay**—HELa and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 1% streptomycin, 1% penicillin, and 1% mycoplasma-free (10 μg/liter). Stable cell lines were generated by transfection of the DNA constructs into HeLa cells using calcium phosphate precipitation. One day before transfection, cells were plated into 9-cm dishes; 5×10^5 cells/dish were transfected with 0.5 μg expression vectors for pFlag-GATA4, pFlag-BAP, pcDNA3-p300HAT, pMT2-GATA4, pMT2GATA4 mutants, pcDNA3-dHAND, and pcDNA3-dHAND mutants, whereas 0.6 μg of CMV-p300 and 0.1 μg of CMV-Id1, CMV-E1A, CMV-E12, and CMV-E2 were used. CMV-Δgalactosidase (20 ng in each well) was used as internal control. Luciferase activity was measured in a luminometer, which was normalized to β-galactosidase activity using Tfx-20 reagent (Promega). The cardiomyocytes were washed with phosphate-buffered saline 14 h post-transfection. The cardiomyocytes were lysed and luciferase activity was measured 24 h post-transfection.

**Electrophoretic Mobility Shift Assays (EMSAs)**—Conditions for EMSA were described previously (37). The DNA GATA-binding site from the
GATA4 and dHAND synergistically activate transcription in cardiomyocytes and HeLa cells. A, rat neonatal cardiomyocytes were transfected with an ANF luciferase promoter (~638 bp) containing reporter plasmid with or without GATA4 and/or dHAND expression vectors. HeLa cells were also transfected with the same reporter under identical conditions. B, HeLa cells were transfected with a BNP luciferase reporter (~116 bp) in the presence or absence of GATA4 and/or dHAND expression vectors. C, HeLa cells were transfected with an α-MHC luciferase reporter (~330 bp) in the presence or absence of GATA4 and/or dHAND. D, Western blot showing that GATA4 and dHAND did not influence each other’s expression in lysates from co-transfected HeLa cells. All results represent triplicate experiments. *, p < 0.05 versus pcDNA3.

ANF promoter) was 5'-CTGATAACTCTGATAACTCTGATAACTG-GTAC, whereas the dHAND-binding site consisted of the sequence 5'-TCGACAGGGCCATCTGGCATTG.

RESULTS

GATA4 and dHAND Synergistically Activate Cardiac Promoters—To gain greater insight into the transcriptional mechanisms whereby GATA transcription factors regulate cardiac-specific gene expression, we surveyed the ability of GATA4 to function in cooperation with the cardiac-enriched bHLH protein dHAND. Promoters from the ANF, BNP, and α-MHC genes were employed for analysis because each was previously shown to require GATA DNA binding activity for cardiac-specific expression (reviewed in Ref. 63). In the presence of both GATA4 and dHAND expression vectors, the ANF promoter showed ~40- and 60-fold induction in transiently transfected neonatal cardiomyocytes and HeLa cells, respectively (Fig. 1A).

Whereas a similar degree of transcriptional synergy was observed in each cell type, HeLa cells lack some cardiac-expressed transcription factors that might otherwise dominantly regulate promoter activity. Indeed, transient transfection of the BNP and α-MHC luciferase fusion constructs into HeLa cells demonstrated a similar degree of synergy in the presence of co-transfected GATA4 and dHAND (Fig. 1, B and C). Importantly, co-transfection of GATA4 and dHAND expression constructs did not result in squelching of either factor compared with individually transfected cells (Fig. 1D). These results indicate that the transcription factors GATA4 and dHAND functionally synergize to enhance expression of the assayed cardiac-expressed gene promoters. Finally, we also observed that the closely related transcription factors GATA5 and GATA6 synergized with dHAND on the ANF promoter, whereas eHAND (HAND1) did not synergize with GATA4 (data not shown).

To analyze the mechanism of this observed transcriptional synergy between GATA4 and dHAND in more detail, multiple deletion constructs were generated and assayed. Deletion of the C terminus of dHAND did not significantly reduce GATA4 synergy on the ANF promoter, suggesting that this domain was dispensable for interaction (Fig. 2A). In contrast, deletion of the N-terminal transactivation domain in dHAND, or both N- and C-terminal domains together, reduced or eliminated transcriptional synergy (Fig. 2A). However, it should be noted that dHAND contains a strong transcriptional activation domain only within its N terminus, suggesting that deletion of this domain could simply reduce the transcriptional potency of any underlying interaction (37). Last, the basic domain of dHAND was directly mutated within the context of the full-length protein, which eliminated all transcriptional synergy (Fig. 2A). Collectively, these results are consistent with the interpretation that GATA4 interacts with dHAND through the bHLH domain to augment transcriptional activation.

To more carefully elucidate the critical interacting domains within GATA4, a similar series of deletion mutants was generated and assayed for functional synergy with dHAND. Deletion of the N-terminal zinc finger domain of GATA4 did not alter transcriptional synergy with dHAND, although deletion of the C-terminal zinc finger domain severely compromised the functional interaction (Fig. 2B). These data indicate that the C-terminal zinc finger of GATA4 is most critical for mediating transcriptional synergy with dHAND.

GATA4 and dHAND Physically Interact in Vitro and in Vivo—The transcriptional synergy observed between GATA4 and dHAND could result from either independent binding of each factor to its cognate site, or from a direct physical interaction. To test this later possibility, glutathione S-transferase (GST) fusion constructs were generated and used for in vitro precipitation experiments. cDNA fragments encoding multiple domains of GATA4 were fused to the GST coding sequence to permit generation of each recombinant protein in bacteria. Each purified fusion protein was loaded onto a glutathione-agarose column and in vitro translated dHAND protein (35S-labeled methionine) was subsequently added to assay for interaction (Fig. 3). The data demonstrate that the C-terminal zinc finger domain of GATA4 strongly interacted with the in vitro translated dHAND, whereas the N-terminal zinc finger domain only showed a minimal interaction (Fig. 3). Comparable quantity of each GST-GATA4 fusion construct was loaded onto GST beads (data not shown), suggesting that the zinc finger domains of GATA4 are capable of physically interacting with dHAND in vitro.

To more carefully elucidate the interactive surface within the C-terminal zinc finger of GATA4, a series of sequential site-
directed mutants was generated for in vitro translation and subsequent interaction with GST-dHAND. All site-directed GATA4 mutant proteins were produced at roughly similar levels by in vitro translation (Fig. 4, top panel). The data demonstrate that each GATA4 mutant was capable of physically interacting with dHAND, except the WRR-SSS mutant, which alters residues at the tip of the C-terminal zinc finger (Fig. 4). GST alone did not interact with any of the mutant or deletion constructs (Figs. 3 and 4). As a further control, deletion of the entire C-terminal zinc finger domain in GATA4 severely attenuated the interaction with dHAND (Fig. 4). Collectively, these results not only confirm the requirement of the C-terminal zinc finger domain for mediating the dHAND interaction, but they also suggest that residues within the tip of the GATA4 C-terminal zinc finger are most critical.

To examine the domain of dHAND that facilitates GATA4 interaction, a series of dHAND deletion constructs were generated for production of in vitro translated protein. Analysis of protein levels showed equivalent amounts of each dHAND deletion protein, except for the bHLH domain, which was only detected at ~10% of the signal of the full-length protein (Fig 5). This decreased signal likely reflects the presence of far fewer methionine residues available for in vitro translation-dependent radioactive labeling compared with the larger frag-
A 20% input

GST

GST-G4 241-378

GATA4 binding

dHAND

dHΔC

dHΔN

bHLH

B-mut

A

FIG. 5. Mapping the domains of dHAND required for GATA4 interaction. A, SDS-PAGE showing the migration of various in vitro translated 35S-labeled dHAND deletion proteins and the basic domain mutant protein (20% input of labeled protein) (upper panel). Each dHAND deletion protein also contained multiple epitope tags, whereas the basic domain mutant (B-mut) lacked epitope tags (gives slightly different migrations). GST alone failed to interact with any of the dHAND deletion proteins (middle panel), whereas a GST-GATA4 fusion protein (amino acids 241–378 corresponding to the C-terminal zinc finger) interacted with all of the dHAND deletion proteins that contained the bHLH domain. However, mutagenesis of the basic domain in dHAND eliminated the interaction. B, the bHLH domain of dHAND interacts with GATA4 in vivo. Expression vectors encoding Flag-GATA4 and Myc-dHAND(bHLH) were co-transfected into HeLa cells, which were subsequently used to generate protein extracts to assay for interaction between these two proteins. The extracts were precipitated with control agarose or FLAG antibody-conjugated agarose and then subjected to Western blotting with anti-Myc antibody. Abbreviations: dH, dHAND; G4, GATA4; N, N-terminal deletion; C, C-terminal deletion.

B Transfect:
Flag-GATA4 +
dH-bHLH-myc

dH-bHLH-myc

FIG. 6. GATA4 and dHAND do not affect each other’s DNA binding activity. A, HeLa cells were transfected with GATA4 alone or GATA4 with dHAND, and protein extracts were incubated with a 32P-labeled GATA DNA-binding site from the ANF promoter and subjected to EMSA analysis. GATA4 antibody and a cold GATA DNA-binding site each blocked the GATA4 mobility shift, whereas co-expression of dHAND had no effect. B, EMSA analysis with in vitro translated dHAND, E12, GATA4, or various combinations using a 32P-labeled dHAND E-box sequence as described previously (37). The E12-dHAND heterodimer band was not affected by in vitro translated GATA4.

GATA4 and dHAND physical interaction—The observed functional synergy between GATA4 and dHAND is presumed to occur through a physical interaction that results in enhanced transcriptional potency. However, it is also possible that the GATA4-dHAND interaction influences the DNA binding activity of one or both factors. To assay for such an effect, EMSAs were performed from transfected HeLa cell extracts in conjunction with a DNA-binding site for GATA4 (Fig. 6A). GATA4 protein efficiently bound to the GATA DNA sequence element, which was efficiently competed by unlabeled oligonucleotide or GATA4-specific antibody. Importantly, GATA4 DNA binding activity was not altered by the presence of co-transfected dHAND. Conversely, the ability of dHAND to recognize an optimized E-box DNA-binding site in conjunction with E12 (37) was also not altered by the presence of in vitro translated GATA4 protein (each factor was generated by in vitro translation in this experiment) (Fig. 6B). It should also be noted that the physical interaction between GATA4 and dHAND was not of sufficient affinity to generate a higher order complex in the mobility shift assay from either co-transfected cells or from in vitro translation of both proteins. However, identification of such higher order complexes using mobility shifts is typically rare given the characteristics of this assay. In any event, these results indicate that the physical
interaction between GATA4 and dHAND does not promote functional synergy through alterations in the DNA binding activities of either factor. On the contrary, these results suggest that functional synergy arises through enhanced transcriptional activation associated with a physical interaction (see below).

GATA4-dHAND Synergy Requires p300—To more carefully examine the mechanism whereby GATA4 and dHAND synergistically activate transcription, artificial reporter constructs specific for each factor was employed. Four multimerized copies of the optimal dHAND E-box sequence element were placed upstream of a TATA-box-containing minimal promoter fused to the luciferase reporter (37). Whereas transfection of a dHAND encoding expression vector only promoted a modest, albeit significant, 2-fold activation of this E-box reporter, co-transfection of GATA4 did not further increase transcriptional activation (Fig. 7A). These data indicate that GATA4 and dHAND do not functionally interact through an E-box DNA-binding site-dependent mechanism. However, reporter constructs containing multimerized GATA sites from either the α-MHC or ANF promoters each demonstrated robust transcriptional synergy between GATA4 and dHAND in transient transfection assays (Fig. 7B). These results suggest that the observed functional synergy between GATA4 and dHAND depends on GATA DNA sequence elements.

The transcriptional synergy between GATA4 and dHAND might simply result from the combined presence of activation domains from each factor. Alternatively, synergy might arise because of recruitment of additional regulatory cofactors such as p300. Interestingly, GATA4 was previously shown to interact with p300 to further augment transcriptional activation (25). To evaluate such a mechanism, transient transfections were performed using the ANF-luciferase reporter and p300 modulatory factors. Co-transfection of the p300 inhibitory protein E1A (encoded by the adenoviral genome) potently blocked GATA4-dHAND transcriptional activation (Fig. 7C). The E1A protein was previously shown to down-regulate expression of muscle-specific genes, presumably by inhibiting p300 (42, 43). In addition, overexpression of the p300 deletion mutant lacking the CH3 interaction domain (missing amino acids 1737–1836) blocked GATA4-dHAND functional synergy (Fig. 7C). The CH3 domain of p300 also mediates interaction with GATA4 and other bHLH-containing transcription factors such as MyoD and NeuroD (25, 26). Taken together, these results indicate that GATA4-dHAND transcriptional synergy requires p300.

Finally, it was also of interest to examine the molecular identity of dHAND involved in transcriptional synergy because dHAND can form both homodimers with itself or heterodimers with ubiquitously expressed E-proteins (37, 44). The ANF-luciferase reporter was co-transfected with GATA4 and dHAND in the presence of either Id1 or E12 (Fig. 7D). The data demonstrate that Id1 had no significant effect on GATA4-dHAND functional synergy, whereas E12 overexpression reduced transcriptional activation (Fig. 7D). Because Id1 only interacts with the E-proteins, it suggests that dHAND homodimers mediate the synergy with GATA4. Consistent with this hypothesis, overexpression of E12, which can complex with dHAND, effectively competed for GATA4-dHAND transcriptional synergy. Taken together, these data suggest that dHAND homodimers mediate transcriptional synergy with GATA4 independent of the ubiquitous bHLH-containing E-proteins.

dHAND Physically Interacts with p300—The observation that p300 was required for mediating GATA4-dHAND synergy suggested that dHAND might also interact with p300, especially because other bHLH-containing factors show a similar relationship (26). Constructs encoding 4 consecutive domains of p300 were in vitro translated and incubated with GST-dHAND to directly evaluate interaction between these factors. The data demonstrate that amino acids 1186–1860 of p300 physically associated with dHAND in vitro, but not with GST alone (Fig. 8A). This domain of p300 contains the CH3 and histone acetyltransferase domains. GST-E1A was employed as an additional control given its well defined ability to interact with a similar domain in p300 (45) (Fig. 8A, right-hand panels). No other domains of p300 were associated with dHAND in this assay (Fig. 8A). We also observed that amino acids 1186–1513 of p300, which contains the histone acetyltransferase domain but lacks the CH3 domain, failed to interact with dHAND suggest-
ing that the CH3 region was most critical (data not shown). Finally, it was also determined that the observed physical association between p300 and dHAND was associated with enhanced transcriptional activation through the dHAND-binding site-dependent artificial reporter (Fig. 8B). Specifically, transient transfection of the E-box luciferase reporter showed synergistic activation in the presence of both dHAND and p300 in HEK 293 cells. Collectively, these results suggest that the CH3 domain of p300 physically associates with dHAND and augments its transcriptional potency.

Finally, it was also of interest to determine the minimal domain of dHAND capable of associating with the p300 CH3 domain. Accordingly, constructs encoding various deletion fragments of dHAND were in vitro translated and incubated with GST-p300CH3 (encodes amino acids 1587–1817). The data demonstrate that every dHAND fragment containing the bHLH domain was capable of interacting with the CH3 domain of p300 (Fig. 9). However, mutation of the basic domain of dHAND nearly abolished p300 interaction, suggesting that the bHLH motif is the minimal domain necessary for mediating a physical interaction with p300.

**DISCUSSION**

GATA4 and dHAND are each expressed in the developing myocardium where they regulate induction of the cardiac gene program and heart maturation. However, neither GATA4 nor dHAND is exclusively expressed in cardiomyocytes, suggesting that cardiac specificity likely arises through a combinatorial code consisting of multiple semi-restricted transcription factors that uniquely overlap in expression in the heart. The observation that p300 also associates with both dHAND and GATA4 complements the notion of a cardiac-specific enhanceosome consisting of multiple semi-restricted DNA-binding factors and global transcriptional effectors as molecular scaffolds.

**Role of dHAND in Regulating Cardiac Transcription**

dHAND is a member of a large transcription factor gene family that contains the bHLH DNA-binding and -dimerization motif.
Other bHLH domain-containing factors have been shown to be master regulators of cell fate and tissue-specific gene expression. For example, the MyoD family of bHLH factors function as direct inducers of skeletal muscle cell specification from mesodermal progenitor cells, as well as their subsequent differentiation (reviewed in Ref. 46). In neuronal cell types, the bHLH proteins Mash1 and NeuroD are involved in determining multiple sublineages in the peripheral and central nervous system (47, 48). However, other bHLH proteins are expressed in a more ubiquitous pattern throughout the body where they function as necessary co-factors in combination with other transcriptional regulatory factors. dHAND is expressed in the developing heart, the limb bud, and multiple neural crest-derived tissues (reviewed in Ref. 49). In the developing mouse heart, dHAND is initially expressed in the embryonic heart tube in a region destined to form the right ventricle. Consistent with its expression pattern, dHAND null mice show a severely atrophic right ventricle, suggesting that dHAND functions as a critical regulator of the right ventricular transcriptional program (36). However, dHAND is unlikely to function as a master regulator of the cardiac lineage analogous to the manner in which MyoD regulates skeletal muscle cell fate given the relatively unrestricted expression pattern of dHAND. Indeed, dHAND plays a critical role in specifying sympathetic neurons, in vascular formation in the developing embryo, in limb development, and in branchial arch development (50–53).

The direct transcriptional targets whereby dHAND participates in regulating diverse cell fates are largely unknown. Most members of the bHLH transcription factor family bind to a DNA sequence element referred to as an E-box, which consists of the loose nucleotide consensus site CANNTG. Whereas dHAND forms both homodimers (44) with itself and heterodimers with the ubiquitously expressed E12/E47 bHLH proteins, only the dHAND/E-protein heterodimers interact with a subset of E-box sequence elements to directly promote transcriptional activation (37). Here we demonstrated that dHAND homodimers function in concert with GATA4 as a mechanism of enhancing cardiac-specific gene expression. This synergy between GATA4 and dHAND was independent of the ability of dHAND to bind DNA and it required a functional interaction with p300. These observations suggest that dHAND can participate in programming the cardiac gene program through combinatorial interactions with other transcription factors, which are likely “bridged” through p300. This model also predicts that dHAND can function in regulating cardiac gene expression independently of the E-box sequence elements and ubiquitous E-proteins such as E12/E47. However, our data do not rule out a potential role for dHAND/E-protein heterodimers as direct transcriptional regulators of target genes through E-box sequence elements.

Role of GATA4 in Regulating Cardiac Transcription—Like dHAND, GATA4 is also thought to function in regulating cell type-specific gene expression through combinatorial interactions with other transcription factors. GATA4 is a member of a highly related subfamily of zinc finger domain-containing transcription factors consisting of GATA4, GATA5, and GATA6. Each of these transcription factors is expressed in a diverse array of cell types throughout development and in the adult vertebrate organism (reviewed in Ref. 54). Most notably, these factors have been implicated in regulating tissue-specific gene expression in the liver, lungs, urogenital ridge, gonads, and heart (reviewed in Ref. 63).

As hypothesized with dHAND, tissue-specific gene regulation mediated through GATA4 likely arises through interactions with other transcription factors that are themselves expressed in semi-restricted patterns. For example, GATA4 physically interacts with the transcription factors dHAND, Nkx2.5, MEF2, SRF, and NFAT, which together are co-expressed only in the myocardium. The combinatorial interaction between GATA4 and Nkx2.5 results in synergistic activation of the ANF promoter and α-actin promoters in cardiomyocytes (18–20). Similarly, GATA4 recruits MEF2 to the ANF promoter to potentiate transcription (22). Interestingly, MEF2 also interacts with p300, collectively suggesting a model whereby each of these interacting factors is part of a larger complex nucleated through p300 (55, 56). It would be nearly physically impossible for GATA4 to simultaneously interact with dHAND, Nkx2.5, MEF2, SRF, and NFAT, given that each factor binds the same C-terminal zinc finger domain of GATA4. However, it is likely that p300/CBP serves as an important transcriptional scaffold to facilitate the formation of cardiac-specific enhanceosome complexes. Consistent with this notion, p300 was previously shown to nucleate a multisubunit complex between forkhead factor and steroid receptor cofactor in the regulation of the insulin-like growth factor binding protein-1 gene (57). p300/CBP also simultaneously interacts with both pax-6 and cdx-2 in the regulation of the glucagon gene promoter (58). Finally, p300 coordinates expression of the lactate dehydrogenase A gene promoter through a multicomponent complex involving hypoxia-inducible factor-1, CAMP-response element-binding protein-1, and other factors (59).

A Cardiac Enhanceosome as the Final Common End Point—Here we showed that the C-terminal zinc finger domain of GATA4 physically associates with the bHLH region from dHAND, which are the same domains that physically associate with p300. Whereas our data suggest a direct physical complex between GATA4 and dHAND, we cannot rule out a bridging effect of p300. Indeed, gel mobility shift analysis of MEF2 DNA binding activity from the programmed reticulocyte lysate identified p300 as part of the shifted complex, indicating the presence of endogenous p300 in this cellular extract system (55). Our assay system involved bacterial generated GST fusion proteins that were incubated with radiolabeled in vitro translated proteins from reticulocyte lysate. This type of assay does not necessarily prove a direct interaction between two factors given the presence of additional co-factors in the reticulocyte lysate. Indeed, the previously characterized interactions between GATA4 and Nkx2.5, SRF, MEF2, and NFAT, which were each mapped to the zinc finger domains of GATA4, utilized programmed reticulocyte lysate. Collectively, these various observations suggest that many of these factors likely interact through indirect mechanisms involving higher order complexes with transcriptional scaffolding molecules. Indeed, the transcription factors signal transducers and activators of transcription 3 and Smad-1 physically interact only through the bridging action of p300 in neuronal progenitor cells (60). In cardiac myocytes, p300/CBP physically associates with GATA4, SRF, MEF2, and NFAT, and now dHAND (25, 55, 56, 61, 62), observations that are consistent with a model of indirect factor association through transcriptional accessory proteins. Whereas our data do not rule out the possibility that GATA4 and dHAND directly interact, we favor the hypothesis of indirect bridging through p300/CBP.

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