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Cooperative Activation of Atrial Naturetic Peptide Promoter by dHAND and MEF2C

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Abstract An intricate array of cell-specific multiprotein complexes participate in programs of cell-specific gene expression through combinatorial interaction with different transcription factors and cofactors. The dHAND basic helix-loop-helix (bHLH) transcription factor, which is essential for heart development and extra embryonic structures, is thought to regulate cardiomyocyte-specific gene expression through combinatorial interactions with other cardiac-restricted transcription factors such as GATA4 and NKX2.5. Here, we determine that dHAND also interacts with the myocyte enhancer binding factor-2c (MEF2C) protein, which belongs to MADS-box transcription factors and is essential for heart development. dHAND and MEF2C synergistically activated expression of the atrial naturetic peptide gene (ANP) in transfected HeLa cells. GST-pulldown and immunoprecipitation assay demonstrate that full-length MEF2C protein is able to interact with dHAND in vitro and in vivo, just like MEF2A and bHLH transcription factors MyoD in skeletal muscle cells. In addition, electrophoretic mobility shift assays (EMSAs) demonstrate that MEF2C and dHAND do not influence each other's DNA binding activity. Using chromatin immunoprecipitation (ChIP) analysis in H9c2 cells we show that dHAND interact with MEF2C to form protein complex and bind A/T sequence in promoter of ANP. Taken together with previous observations, these results suggest the existence of large multiprotein transcriptional complex with core DNA binding proteins that physically interact with other transcriptional factors to form favorable conformation to potentiate transcription. J. Cell. Biochem. 93: 1255–1266, 2004.

Key words: synergism; ANP; transcription; dHAND gene; MEF2C gene

The regulation of tissue specification and determination involves a network of regulatory factors that modulate the activity of target genes. In cardiac development, multiple transcription factors, including dHAND, myocyte enhancer factor-2C (MEF2C), GATA4, and NKX2.5, play important roles [Olson and Srivastava, 1996; Srivastava, 1999]. dHAND is a basic helix-loop-helix (bHLH) transcription factor. It is expressed in mesodermal and neural crest-derived structures during heart development. If lacking dHAND during mouse embryogenesis, heart development would be disturbed and would not progress beyond the cardiac-looping stage [Hollenberg et al., 1995; Srivastava et al., 1995, 1997].

The bHLH proteins contain the basic region and the HLH domain, the basic region can bind to the E-box consensus sequence (CANNTG) and the HLH domain can mediate homodimerization or heterodimerization with itself or with

Abbreviations used: bHLH, basic helix-loop-helix; ANP, atrial naturetic peptide gene; MEF2C, myocyte enhancer factor-2C; MADS-box, (MCM1, Agamous, Deficiens, Serum response factor)-box; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; α-MHC, a-myosin heavy chain; AA, amino acid; PCR, polymerase chain reaction; CMV, cytomegalovirus; TNT, transcription and translation; EMSA, electropheoretic mobility shift assay; ChIP, chromatin immunoprecipitation assay.

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E proteins such as E12. In addition, the bHLH proteins have also been shown to form complex with non-bHLH protein such as the myocyte enchancer factor-2. For example, in skeletal muscle cells, the muscle bHLH protein MyoD form complex with MEF2A, a member of myocyte enchancer factor-2 (MEF2) family to cooperatively active skeletal muscle genes and physically interact through the MADS domain of MEF2A [Kaushal et al., 1994].

MEF2C is another transcription factor which controls cardiac morphogenesis and myogenesis. It belongs to MEF2 family of MADS (MCM1, Agamous, Deficiens, Serum response factor)-box. In mammals, MEF2 family is composed of four members, MEF2A, MEF2B, MEF2C, and MEF2D. They can bind the consensus DNA A/T rich sequence (T/C)TA(A/T)$_4$TA(G/A) present in the control region of numerous muscle-specific genes by forming homodimers or heterodimers. Members of the MEF-2 family share homology in an N-terminal 56 amino acid MADS domain and an adjacent 29 amino acid MEF2 domain, which together mediate DNA binding and dimerization [Martin et al., 1993].

In cardiac myocytes, dHAND and MEF2C are both thought to play particularly important roles in regulating most cardiac-specific gene expression, including atrial naturetic peptide gene (ANP) and a-myosin heavy chain (α-MHC) [Adolph et al., 1993; Dai et al., 2002]. Each of them directly or indirectly interact with other tissue-specific transcription factors to control cardiac gene expression. Transcription of cardiac genes is mediated by the interaction of specific transcription factors such as dHAND and GATA4 [Dai et al., 2002], dHAND and NKX2.5 [Miano, 2002; Thattaliyath et al., 2002], MEF2C and GATA4 [Morin et al., 2000], but whether dHAND and MEF2C can interact with each other to regulate a subset of cardiac muscle-specific genes expression has not yet been examined.

On the other hand, the MADS-box transcription factor MEF2C is expressed throughout the heart during mouse embryogenesis, MEF2C mutant will lead to defects in cardiac looping and the right ventricle. Moreover, a subset of cardiac muscle gene will not be expressed [Lin et al., 1997]. The cardiac phenotype of the MEF2C mutant is similar to that of the dHAND mutant, suggesting that they regulate each other's expressions in the future right ventricle region or that these two transcription factors cooperate to control cardiac-specific target gene expression, as has been described for MEF2A and myogenic bHLH MyoD protein cooperatively active skeletal muscle genes in the skeletal muscle lineage [Lin et al., 1997].

All these prompt us to address whether dHAND and MEF2C can synergistically active cardiac-specific target gene expression and physically interact with each other to potentiate transcription. We used a promoter derived from the ANP gene. As a cardiac-specific gene, ANP is highly restricted to this organ during heart development with the highest level of expression in the right ventricle. Previously, a 0.7 kb upstream fragment of the ANP gene was sufficient for cardiac-specific gene expression. This fragment harbors a number of DNA-binding elements of many general and cardiac-specific transcription factors, So ANP serve as a marker gene and can be used to study the regulatory mechanisms whereby control cardiac gene expression [Durocher et al., 1997; Habets et al., 2002]. We present data showing that dHAND and MEF2C physically interact with each other both in vitro and in vivo, they form protein complex through interaction and bind to A/T rich sequence to cooperatively activate the cardiac-restricted ANP promoter. This molecular interaction provides further insight into the mechanisms whereby the complex interactions among transcription factors and target DNAs fine-tune gene expression in cardiomyocyte.

MATERIALS AND METHODS

Plasmid Construct

The pcDNA3-MEF2C expression vector was a gift of Dr. Coralie Poizat and Larry Kedes (California, CA). The pFLAG-GATA4 expression vector, pcDNA3-His2B-dHAND expression vector, pGEX2TK-dHAND expression vector, the ANP reporter plasmid (−638 base pairs upstream from the transcriptional start site) were gifts from Dr. Jeffery D.Molkentin and Yan-Shan Dai (Cincinnati, OH). pFLAG-MEF2C was generated by subcloning MEF2C PCR fragment from pcDNA3-MEF2C using primer P1 and P5 into the *Hind*III and *Xba*I sites in pFLAG-CMV-2. These primer were: P1 5′ATCCCAAGCTTATGGGGAGAAAAAAGATTCAG3′; P5 5′GCTAGTCTAGATTATGTTGCCCATCCTTCAGA 3′. P1 primer was simulta-

neously added *Hind*III restriction site at the start of translation codon, P5 primer was simultaneously added *Xba*I restriction site after stop codon.

Transient Transfection Assay

Hela cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, streptomycin (10 g/L) and penicillin (10 g/L). All transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol in six-well plates. Twenty-four hours after plating, each well were transfected with 0.3 μg of reporter DNA ANP-Luc, and 0.3 μg of expression vectors pcDNA3-MEF2C or/and pcDNA3-HisB-dHAND. β-galactosidase (20 ng in each well) was used as internal control, the total amount of DNA was kept constant using pcDNA3.1 empty vector. Luciferase activity was measured 48 h after transfection in a luminometer using FOLAR star reporter assay system (BMG) and normalized to β-galactosidase activity. Each value presented is the average of triplicate samples and representative of multiple independent experiments (n greater than or equal to 3). The date were statistically analyzed with a Student's *t* test.

Immunoprecipitation and Western Blot Analysis

Hela cells were transfected with pFLAG-MEF2C and pcDNA3-His-dHAND. The cells were lysed at 4°C with gently rotation 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 12,000*g* for 10 min. Lysate proteins were immunoprecipitated overnight at 4°C with anti-FLAG M2-affinity gel (Sigma, St. Louis, MO) by gentle rotation. The agarose was washed and the bound proteins were eluted with sample buffer and subjected to SDS–PAGE. Resolved proteins were transferred onto Hybond-PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and Western blotted. The blot was incubated with mouse anti-His antibody (Clontech, Palo Alto, CA) and detected with ECL chemiluminescence reagents (Santa Cruz, Santa Cruz, CA) following the recommended protocol. MEF2C polyclonal antibody was purchased from Santa Cruz.

GST Pull-Down Assay

The expression vector for glutathione S-transferase (GST)-dHAND fusion protein, pGEX2TK-dHAND, was transformed into *Escherichia coli* BL21 cells. The bacterial cultures were induced by 1 mM isopropyl-D-thiogalactopyranoside for 5 h at 37°C. The bacteria were harvested and sonicated in phosphate-buffered saline containing protease inhibitors (as above). The bacterial lysates were brought to 1% Triton and incubated for 1 h at 4°C then centrifuged at 12,000*g* for 15 min. GST fusion protein were purified by binding to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) at 4°C for 2 h before washing three times in phosphate-buffered saline containing protease inhibitors. The concentration of proteins immobilized on beads was estimated by SDS–PAGE and quantified against bovine serum albumin standards (Sigma) after Coomassie Blue staining. Binding assays were performed with GST-dHAND fusion protein and in vitro translated MEF2C protein using a TNT-coupled reticulocyte lysate system (Promega, Madison, WI) in the presence of ^{35}S-labeled methionine (Amersham Pharmacia Biotech). Fifteen microliters of ^{35}S-labeled MEF2C proteins and equal amounts of immobilized GST or GST fusion proteins were incubated for 2 h at 4°C with gentle rotation in GST binding buffer (40 mM Hepes, pH 7.2, 50 mM Na acetate, 150 mM NaCl, 2 mM EDTA, 5 mM dithiothreitol, 0.5% Nonidet P-40, protease inhibitors, and 2 mg of bovine serum albumin per ml). After four times washes in GST binding buffer, beads were resuspended in SDS sample buffer, boiled and resolved by SDS–PAGE, and analyzed by autoradiography.

Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotide pairs were resuspended and boiled for 10 min, and annealed by slowly cooling to room temperature. Annealed oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP (50 μCi), purified with QIAquick Nucleotide Kit following the recommended protocol. For each EMSA assay, 5 μl of each in vitro translated protein lysate or unprogrammed reticulocyte lysate was preincubated for 30 min at 37°C then mixed with binding buffer (50 mM Tris-HCL, pH 7.6, 80 mM NaCl, 8% glycerol, 0.25 μg poly(dI:dC),

5 mM $MgCl_2$, 0.5 mg /ml BSA, 0.6 mM DTT containing protease inhibitors) at room temperature for 20 min; then ~0.1 pmol of radiolabeled oligonucleotide probe was added and incubated for 1 h at 4°C in a 10 μl total volume. Unlabeled double-strand oligonucleotides were added as competitors at 50, 100, or 200-fold molar excess when required. Binding reactions were analyzed on a 5% native polyacrylamide gel in 0.5× Tris borate-EDTA.The gel was dried and exposed to X-ray film for autoradiographic analysis. The MEF2C DNA-binding site (A/T-rich sequence from the rat ANP promoter) was: 5′ GATCCATACTCTAAAAAAATATAATAGCTCTTTCA 3′, mutated A/T-rich sequence was: 5′GATCCATACGACGTCTCGGCTGAATAGCTCTTTCA3′, whereas the dHAND DNA-binding site consisted of the sequence: 5′ TCGACAGGGCCATCTGGCATTG 3′ [Dai et al., 2002], mutated dHAND-binding sequence was: 5′TCGACAAAATTCGTAAACATTA 3′.

Chromatin Immunoprecipitation (ChIP) Assay

H9c2 Cells were grown to 95% confluence in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Cells were collected and cross-linked with 1% formaldehyde at 37°C for 10 min, then were rinsed with ice-cold PBS twice and centrifuged for 5 min at 2,000*g*. Cells were then resuspended in 0.2 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl, pH 8.1, protease inhibitor) and sonicated twelve times for 10 s each, followed by centrifugation for 10 min. Supernatants were collected and diluted in buffer (1.1% Triton X-100, 0.01% SDS, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.1) followed by immunoclearing with 80 μl salmon sperm DNA/protein A agarose for 2 h at 4°C. Immunoprecipitation was performed overnight at 4°C with dHAND antibody (Santa Cruz) or MEF2C antibody. After immunoprecipitation, 60 μl salmon sperm DNA/protein A agarose was added and the incubation was continued for another 1 h. Precipitates were washed sequentially for 5 min each in low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and LiCl immune complex wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA,10 mM Tris-HCl, pH 8.1). Precipitates were then washed two times with TE buffer, then were used to do immunoblot assay for MEF2C protein or polymerase chain reaction (PCR) assay for their binding sequence. If for PCR assays, precipitates were extracted three times with 1% SDS, 0.1 M $NaHCO_3$. Eluates were pooled and heated at 65°C for at least 6 h to reverse the formaldehyde cross-linking, DNA fragments were recovered by phenol/chloroform extraction and ethanol precipitation. One microliter from 50 μl DNA extraction and 25–30 cycles of amplification for PCR were used. ChIP assay kit was purchased from Upstate. The primer for E-box is: sense primer 5′ TCCACCCACGAGGCCAATGAAT 3′; antisense primer 5′ CCGCTCGAGGATGTTTGCTGTCTCGGCTC 3′. The primer for A/T site is: sense primer 5′ GACCTGTATCATGTTGGCTTCCTGG 3′, antisense primer 5′ATTGGCCTCGTGGGTGGACCTCTGG 3′.

RESULTS

Synergistic Activation of the ANP Promoter by dHAND and MEF2C

To begin search for dHAND and MEF2C regulatory elements within the rat ANP promoter, we employed Genbank databases to identify these *cis*-elements of ANP ranging from nucleotide −636 to the transcriptional start site (Fig. 1A). These analysis revealed this fragment contains four E-box and one A/T rich sequence.

Based on previous studies, the basic region of dHAND can bind to the E-box consensus sequence (CANNTG) while MEF2C can bind the consensus DNA A/T rich sequence (T/C)TA(A/T)$_4$TA(G/A) present in the control region of numerous muscle-specific genes [Martin et al., 1993; Dai and Cserjesi, 2002], they all regulate cardiac promoter and are expressed in cardiomyocyte. To gain greater insight into the transcriptional mechanisms whereby dHAND and MEF2C transcription factors regulate cardiac-specific gene expression, we investigated whether dHAND and MEF2C could functionally interact at the promoter level of the ANP gene. Co-transfection of dHAND and MEF2C expression constructs in Hela cells lead to a ~13-fold increase in synergistic activation of the ANP promoter (Fig. 1B), while co-transfection dHAND and GATA4 or and NKX2.5 can result in ~11-fold or ~8-fold induction of the ANP promoter. These results suggest that the ANP gene is synergistically activated by co-

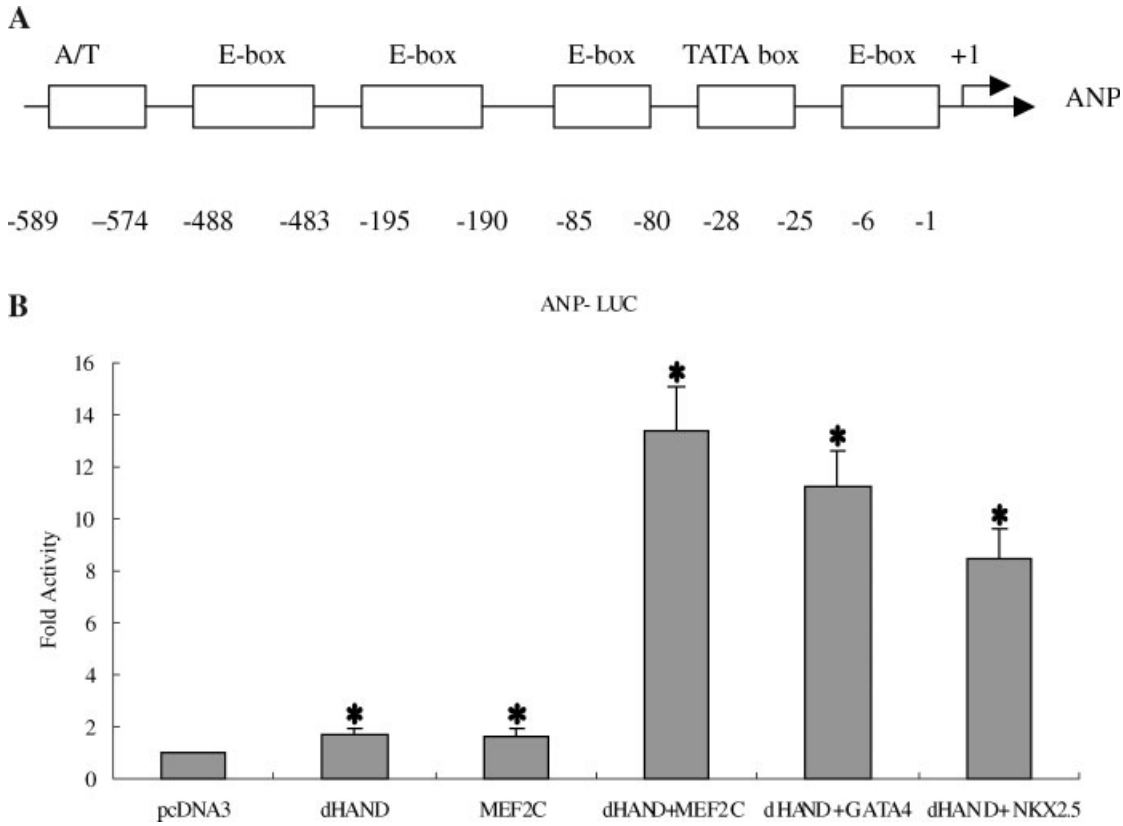

Fig. 1. **A**: Diagram of regulatory elements of dHAND and myocyte enhancer factor-2C (MEF2C) in the atrial naturetic peptide (ANP) promoter. The ANP promoter harbors four E-box and an A/T rich sequence. **B**: dHAND and MEF2C synergistically active ANP transcription in HeLa cells. HeLa cells were transfected with an ANP luciferase reporter (−638 bp) in the presence or absence of dHAND and/or MEF2C expression vector. dHAND + GATA-4, dHAND + NKX2.5 serve as positive control. All results represent at least four times experiments. *Denotes statistical significance at a level of confidence $P < 0.05$ versus pcDNA3. Error bars denote standard deviation. Data as mean ± SE for dHAND, MEF2C, dHAND + MEF2C, dHAND + GATA4, dHAND + NKX2.5 is 1.73 ± 0.18, 1.6 ± 0.36, 13.4 ± 1.4, 11.1 ± 1.2, 8.45 ± 1.7.

existed dHAND and MEF2C transcription factors. Importantly, co-transfection of MEF2C and dHAND expression vectors did not result in squelching of either factor compared with individually transfected cells (Fig. 2). Collectively, these results indicate that the transcription factors dHAND and MEF2C can functionally synergize to activate the ANP promoter.

dHAND and MEF2C Physically Interact In Vitro and In Vivo

The transcriptional synergy observed between dHAND and MEF2C probably result from a direct physical interaction, or from an independent binding to a functional complex (enhanceosome), respectively, by bridging through other coactivators, adaptors, or transcription factors [Dai and Markham, 2001; Vo and Goodman, 2001]. To address this issue, GST pull-down assay was performed with GST-dHAND and in vitro translated MEF2C protein. GST-dHAND immobilized on glutathione-Sepharose beads could retain in vitro translated full-length MEF2C, whereas GST alone could not bind to MEF2C, indicating that dHAND directly interacts with full-length MEF2C in vitro (Fig. 3).

To examine whether dHAND and MEF2C directly interact with each other in vivo, co-immunoprecipitation experiment was performed. Hela cells were transiently transfected with His-tagged dHAND and FLAG-tagged MEF2C expression vector. FLAG-agarose was used to precipitate MEF2C from protein lysates, which was resolved in SDS–PAGE and subjected to Western Blot with anti-His antibody to detect the dHAND protein. The data demonstrate that MEF2C interact with dHAND in vivo, but not with unconjugated agarose alone (Fig. 4). These data suggest that MEF2C and dHAND physically interact in vivo.

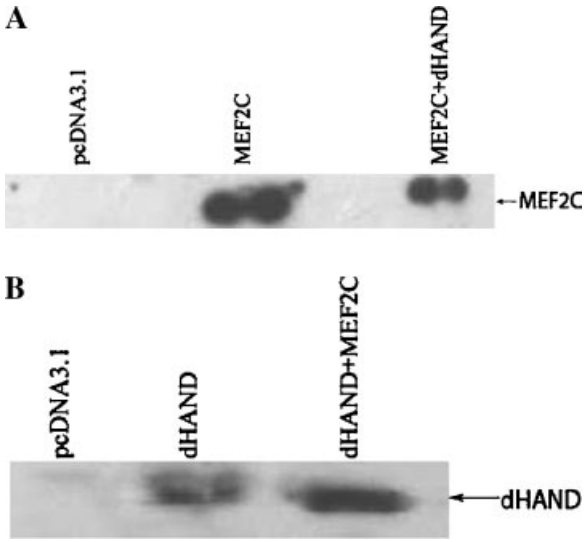

Fig. 2. Western blot assays showing that MEF2C and dHAND do not squelch each other's expression in lysates from co-transfected HeLa cells. Protein lysates from transfected HeLa cells were run through SDS–PAGE, then transferred onto Hybond-PVDF membrane and Western blotted. The blot was incubated with antibody and detected with ECL chemiluminescence reagents. **Panel A**: Nuclear extract from HeLa cells transfected with pcDNA3.1, or pcDNA3.1 and MEF2C, or dHAND and MEF2C were used for Western blot assay with MEF2C-specific polyclonal antibody. **Panel B**: Protein lysates from transfected HeLa cells with pcDNA3.1, or pcDNA3.1 and dHAND, or dHAND and MEF2C were used for Western blot assay with anti-his antibody.

MEF2C and dHAND do not Influence Each Other's DNA Binding Activity

The functional synergy between MEF2C and dHAND may occur through a physical interaction to enhance transcriptional potency. On the other hand, the interaction between MEF2C and dHAND might increase each other's DNA binding affinity to active transcription [Herschlag and Johnson, 1993; Carey, 1998]. To address this issue, EMSAs were performed to examine the DNA-binding activities of MEF2C and dHAND which were synthesized in vitro using TNT-coupled rabbit reticulocyte system (Fig. 5) in the presence or absence of each other. MEF2C and dHAND were efficiently bound to their DNA sequence element, which were efficiently competed with unlabeled oligonucleotide and were not affected by the mutant

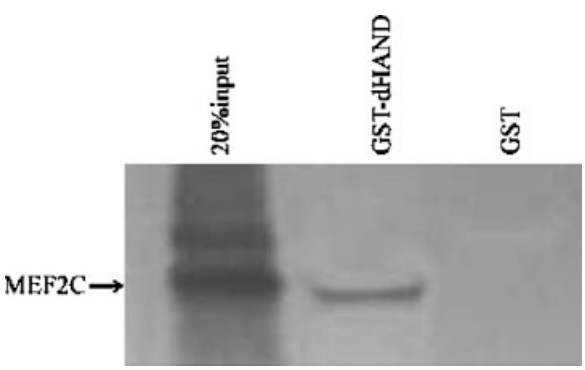

Fig. 3. GST pull-down assay showing that dHAND and MEF2C physically interact in vitro. ^{35}S-labeled MEF2C (20% input of labeled protein) were incubated with GST protein alone or GST-dHAND, then the protein complexes were washed and separated on 10% SDS–PAGE, dried, and analyzed by autoradiography. Result showing that MEF2C could bind dHAND but GST protein alone failed to interact with MEF2C.

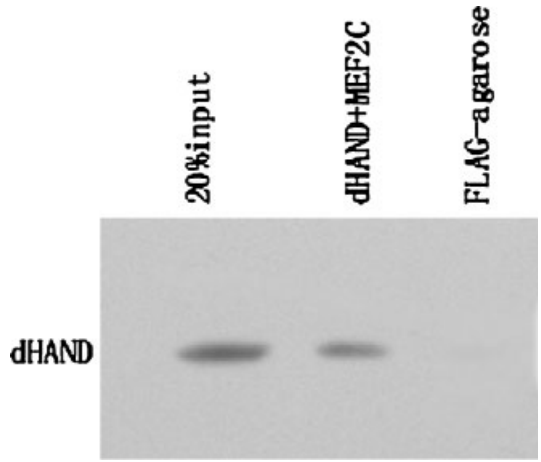

Fig. 4. Immunoprecipitation assay showing that MEF2C and dHAND directly interact in vivo. His-tagged dHAND was co-transfected FlAG-tagged MEF2C in HeLa cells. Lysates were immunoprecipitated with FLAG conjugated agarose beads. Protein lysates, immunoprecipitated protein complex, and FLAG-agarose were then run through 10% SDS–PAGE, transferred onto Hybond-PVDF membrane and incubated with anti-His antibody. Result showing that MEF2C and dHAND directly interact in vivo.

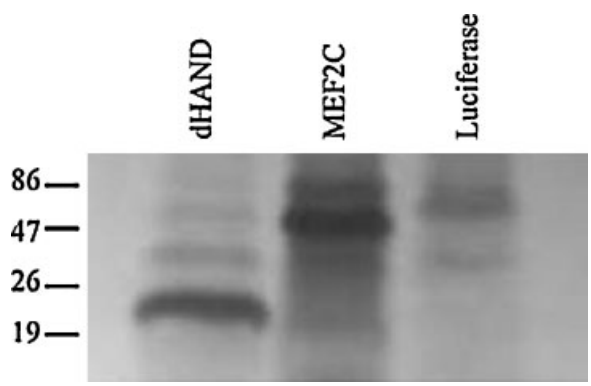

Fig. 5. SDS–PAGE showing the migration of various in vitro translated ^{35}S-labeled proteins. They are dHAND, MEF2C, and luciferase. In vitro translated dHAND and MEF2C were used in EMSA (Figs. 6 and 7) experiment.

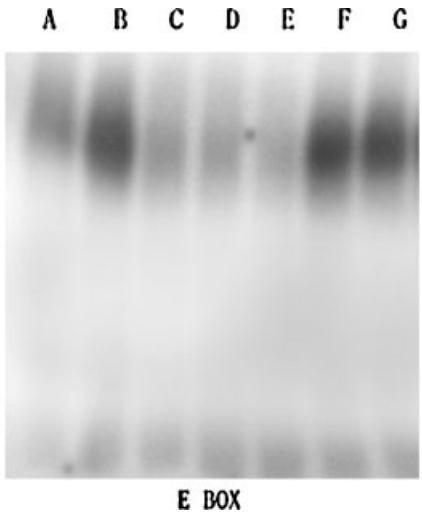

Fig. 6. EMSA assay showing dHAND efficiently bind E-box sequence and MEF2C do not influence DNA-binding activity of dHAND. In vitro translated dHAND was incubated with ^{32}P-labeled or not labeled (compete) oligonucleotides containing E-box site or mutant oligonucleotides, then the incubated mixture were separated on a 5% native polyacrylamide gel in 0.5× Tris borate-EDTA. Then the gel was dried and autoradiography. The result showing that the E-box is a high affinity binding element of the dHAND protein, competed oligonucleotides dramatically decreased the binding affinity of dHAND. When in vitro translated dHAND protein and MEF2C protein were incubated with ^{32}P-labeled oligonucleotides, the DNA-binding activity of dHAND is not altered. **Lane A**, unprogramed; **lane B**, dHAND; **lane C**, dHAND compete (50×); **lane D**, dHAND compete (100×); **lane E**, dHAND compete (200×); **lane F**, dHAND mutate; **lane G**, dHAND + MEF2C.

Fig. 7. EMSA assay showing MEF2C efficiently bind A/T element of the ANP promoter and dHAND do not influence DNA-binding activity of MEF2C. EMSA experiments were performed as described above (Fig. 6). The result demonstrates that competed oligonucleotides dramatically decrease the binding affinity of MEF2C, but the MEF2C band is not affected by in vitro translated dHAND. **Lane A**, unprogramed; **lane B**, MEF2C; **lane C**, MEF2C + dHAND; **lane D**, MEF2C compete; **lane E**, MEF2C mutate.

sequence (Figs. 6 and 7). Importantly, the ability of dHAND to recognize the E-box DNA-binding site [Dai and Cserjesi, 2002; Dai et al., 2002] was not altered when the MEF2C protein coexisted (Fig. 6). Meanwhile, the DNA-binding activity of MEF2C was neither enhanced nor reduced by the presence of dHAND protein (Fig. 7). These results suggest that the physical interaction between MEF2C and dHAND is able to promote functional synergy through enhancing transcriptional potency but not through increasing each other's DNA binding activities.

dHAND and MEF2C can Form Protein Complex and Bind to A/T Sequence of the ANP Promoter in H9c2 Cells

Based on EMSA assays, we may propose a possible model about transcriptional synergy between dHAND and MEF2C. The model is that dHAND and MEF2C each binds its DNA-binding sites and interact with each other by DNA bending through other coactivators to augment their transcriptional potency. But the EMSA assay system is in vitro, it is possible that binding one or other of these transcription factors is altered in the context of the full promoter, so we do not rule out the other possible model whereby MEF2C–dHAND protein complex bind to one of the E-box and A/T sequence element to enhance their transcriptional potency. To address these issues, a serial of ChIP experiments were performed in H9c2 cells which are derived from embryonic rat heart. First, soluble chromatin from H9c2 cells was immunoprecipitated with dHAND antibodies followed by release of the immune complexes and immunoblotted with MEF2C antibodies. For a negative control, perform a no-antibody immunoprecipitation (Fig. 8A).

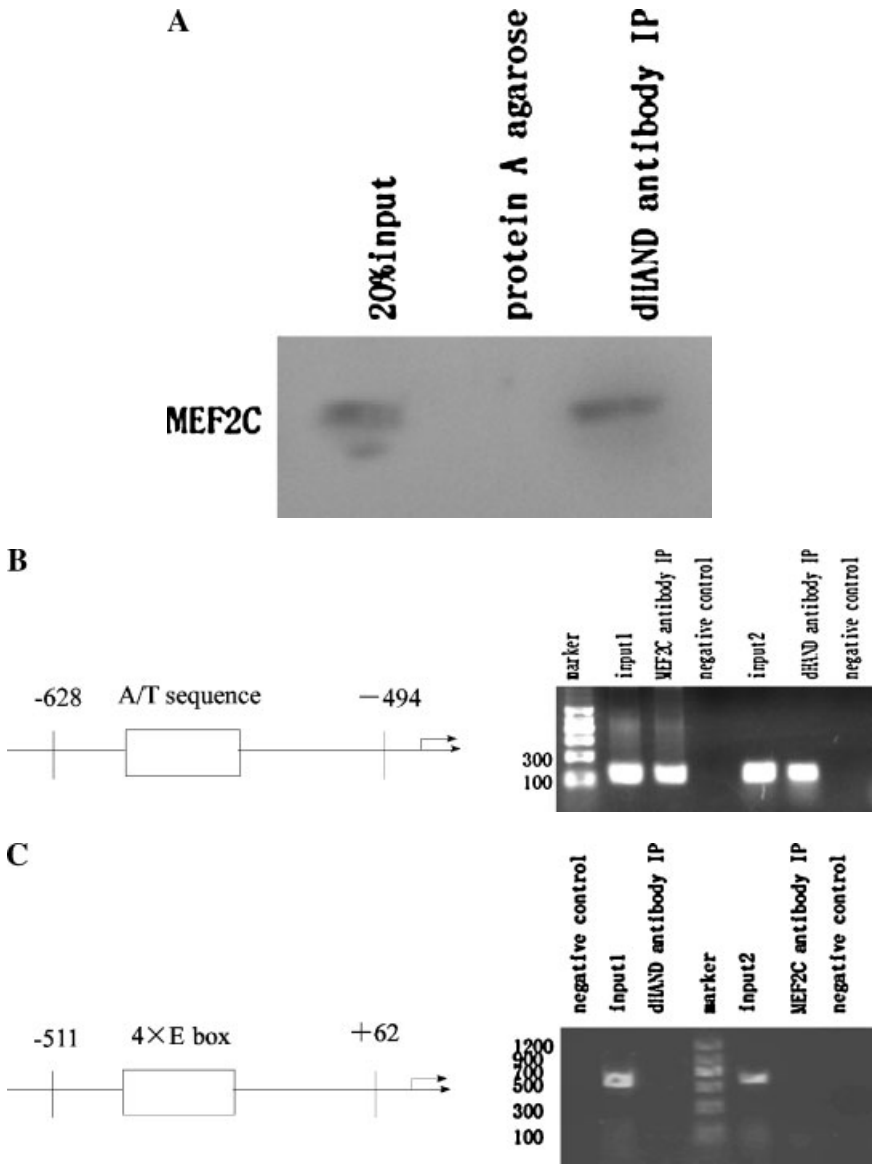

Fig. 8. Chromatin immunoprecipitation (ChIP) assay showing that dHAND and MEF2C form protein complex through interaction and bind A/T rich sequence of the ANP promoter in H9c2 cells. **Panel A**: ChIP to examine whether dHAND and MEF2C can form protein complex in H9c2 cells. Anti-dHAND antibody immunoprecipitated chromatin from lysates of H9c2 cells. The immunoprecipitate was resolved by SDS–PAGE, transferred onto Hybond-PVDF membrane and probed with anti-MEF2C antibody. Proteins were visualized using the ECL chemiluminescence detect system. Result showing that dHAND could interact with MEF2C but protein A agarose alone failed to interact with MEF2C. **Panel B**: Occupancy of the A/T element of ANP promoter by dHAND and MEF2C was measured by ChIP. Soluble chromatin from H9c2 cells was immunoprecipitated with antibodies against dHAND or against MEF2C. The final DNA extractions were amplified using pairs of primers that cover the region of A/T rich sequence of ANP promoter as indicated. The result showing both dHAND and MEF2C are assembled on this region. **Panel C**: ChIP experiment to examine whether the dHAND–MEF2C complex are assembled on the E-box sequence of the ANP promoter. ChIP experiments were performed as described above (B), DNA extractions were amplified using primers which cover the region of E-box element of the ANP promoter.

The result demonstrates that dHAND and MEF2C can form protein complex in H9c2 cell, which is consistent with our result of co-immunoprecipitation assay in HeLa cell. To further examine dHAND–MEF2C protein complex are assembled on one or both sites, we divided the soluble chromatin into two aliquots, one was immunoprecipitated with dHAND antibodies and the other with MEF2C antibodies, both of the eluted immunoprecipitates were amplified both sites sequence of ANP promoter (Fig. 8B,C). The data demonstrate

that both dHAND and MEF2C can occupy the DNA fragment from −628 to −494 bp which harbors A/T sequence, while they all don't assembled on the DNA fragment from −511 bp to +62 bp which harbors E-box sequence. All these results demonstrate that dHAND and MEF2C can form protein complex and bind to A/T sequence of the ANP promoter in H9c2 cell.

DISCUSSION

In this study, we have obtained the following results. (1) dHAND and MEF2C synergistically activate ANP promoter in Hela cells. (2) dHAND and MEF2C directly interact with each other both in vivo and in vitro. (3) EMSA assays suggest that dHAND and MEF2C do not alter each other's DNA-binding affinity. (4) ChIP experiments demonstrate that dHAND and MEF2C can form protein complex and bind to A/T sequence of the ANP promoter in H9c2 cell.

dHAND and MEF2C Synergistically Activate the ANP Promoter

As key regulators of cardiac myogenesis and morphogenesis, dHAND and MEF2C are both expressed during heart development where they participate in a highly orderly complex network of combinatorial interactions to regulate cardiac gene expression. However, whether these two transcription factors cooperate to control heart development is still unknown. These data presented here provide evidence that, in Hela cells, MEF2C and dHAND protein synergistically activate the ANP promoter. Importantly, co-transfection of dHAND and MEF2C expression vectors do not squelch each other's expression compared with individually transfected cells. This result demonstrates that the transcription factors dHAND and MEF2C functionally synergize to activate transcription of the ANP promoter. This observation is reminiscent of the cooperative interaction between MEF2 proteins and the myogenic bHLH factor MyoD in skeletal muscles. These myogenic bHLH factors are only expressed in skeletal muscle, not in cardiac muscle. Whereas dHAND, being a bHLH transcription factor, was found to be expressed in the heart during development [Kaushal et al., 1994; Srivastava et al., 1995, 1997]. Thus, there is probably a model wherein the MEF2 family participates in physically interaction with cell-specific bHLH transcriptional regulators to synergistically activate target gene expression.

Co-transfection of dHAND and MEF2C exhibits transcriptional synergy of ANP promoter and augment luciferase activity over 13-fold in our system. On the other hand, co-transfection both dHAND and GATA4 expression vectors or both dHAND and NKX2.5 expression vectors lead to a ~11-fold or ~8-fold synergistic induction of the ANP promoter under our assay conditions. But Dai et al. [2002] had reported that the ANP promoter showed ~60-fold induction when co-transfected GATA4 and dHAND, Thattaliyath et al. [2002] had reported that together NKX2.5 and dHAND resulted in ~20-fold activation. These suggest that in our system co-transfection dHAND and MEF2C, dHAND and GATA4, or dHAND and NKX2.5 all result in modest synergistic increase in ANP transcriptional activity. The reason is not clearly yet, but the synergy either between dHAND and GATA4 or between dHAND and NKX2.5 serves as an important control and suggests that the observed synergy between dHAND and MEF2C is not due to non-specific effects.

Molecular Mechanism for MEF2C and dHAND Cooperation on the ANP Promoter

GST pull-down assay and co-immunoprecipitation experiments demonstrate that MEF2C and dHAND directly interact with each other both in vivo and in vitro. These suggest that the transcriptional synergy observed with MEF2C and dHAND is via direct protein–protein interaction between MEF2C and dHAND, not independent binding to a functional complex (enhanceosome).

Based on the recent reports of dHAND and MEF2C DNA binding analysis [Morin et al., 2000; Dai and Cserjesi, 2002; Dai et al., 2002], we performed EMSAs analysis on the A/T-rich element of ANP promoter and dHAND binding E-box sequence $(G)_3$CCATCTGG. Here, we show that dHAND is able to bind E-box, When the MEF2C protein co-existed, the E-box-binding affinity of dHAND is not affected, suggesting that the interaction between MEF2C and dHAND do not promote dHAND to efficiently bind E-box. Moreover, this also demonstrates that MEF2C and dHAND forms a specific protein complex through other cofactors to potentiate transcription.

The DNA-binding affinity of MEF2C is not alter by the presence of in vitro translated dHAND protein. These result indicate that the physical interaction between dHAND and

MEF2C promote functional synergy through enhancing transcriptional potency but not through alterations in the DNA binding activities of each factor.

EMSA assays show that dHAND and MEF2C can bind to their respective elements whether alone or together. But only the EMSA assay can not enable us to understand molecular mechanism for MEF2C and dHAND cooperation on the ANP promoter. Because EMSA assay system is in vitro, which may be different from the system in vivo. In vivo, these binding may be altered in the presence of full promoter. So we did a serial of ChIP experiments to determine interaction between MEF2C and dHAND take place at one or both of the sites. H9c2 cells, which derived from rat embryonic heart, were employed for ChIP experiment because ANP, dHAND, and MEF2C are endogenously expressed in H9c2 cells. ChIP assays demonstrate that dHAND and MEF2C can form protein complex and bind to A/T sequence of the ANP promoter in H9c2 cells. So the interaction between MEF2C and dHAND take place at A/T site. All these results suggest that the molecular mechanism for MEF2C and dHAND cooperation on the ANP promoter is involved that dHAND–MEF2C protein complex combined with A/T sites and interact with the basal transcriptional complex to form stable enhanceosome to potentiate transcription.

From our ChIP experiment, we provide direct evidence that the synergy between dHAND and MEF2C is independent of the DNA binding of dHAND and that interaction can occur on MEF2C-binding site. Therefore, dHAND and MEF2C can active transcription via the domain of DNA-binding MEF2C as well as recruitment of dHAND by MEF2C into a cardiac-specific transcriptional active complex (enhanceosome). Consistent with our results, Thattaliyath et al. [2002] had reported that the ability of dHAND to function as a transcriptional activator may be partially independent of DNA binding. Their careful studies suggest that dHAND effects heightened ANP promoter activity is through its interaction with other transcription factors such as NKX2.5 which can directly contact ANP promoter elements not through its binding to target gene.

Indeed, this is a kind of model about transcriptional synergy on ANP. For example, NKX2.5 recruit dHAND [Thattaliyath et al., 2002], NKX2.5 recruit GATA4 [Shiojima et al., 1999], GATA4 recruit MEF2C [Morin et al., 2000], and now we demonstrate that MEF2C recruit dHAND to the ANP promoter to activate transcription. All together, the temporal and spatial regulation of these interactions control cardiac morphogenesis and myogenesis during heart development.

Roles of dHAND and MEF2C in Regulating ANP Transcription and Cardiac Enhanceosome

dHAND and MEF2C are each expressed in the developing myocardium where they regulate induction of the cardiac gene program and heart maturation. dHAND is expressed in deciduum, heart, autonomic nervous system, and neural crest derivatives. Thattaliyath et al. [2002] demonstrate that dHAND directly activates the promoter of ANP in heterologous HEK293 cells. Using whole mount in situ hybridization, they showed that the levels of ANP mRNA were virtually abolished in the hearts of dHAND null mice [Thattaliyath et al., 2002]. So ANP is a downstream target gene for dHAND and its transcription is regulated by dHAND.

Similarly, transcription factor MEF2C control of cardiac morphogenesis and myogenesis. It is expressed in heart precursor cells before formation of the linear heart tube. In the MEF2C null mice, a subset of cardiac specific gene such as ANP, cardiac α-actin, and α-myosin heavy chain were not expressed [Lin et al., 1997]. This indicates that ANP is a downstream target gene for MEF2C. Accordingly, ANP is a common downstream target gene for transcription factors dHAND and MEF2C. In addition to dHAND and MEF2C, the transcription factors GATA4, AP1, SRF, YY1, and NKX2.5 have also been shown to regulate ANP transcription [Thattaliyath et al., 2002]. We further demonstrate that dHAND and MEF2C form protein complex through interaction and bind A/T rich sequence of the ANP promoter to cooperatively activate the cardiac-restricted ANP gene in H9c2 cells.

Our results, considering previous findings [Durocher et al., 1997; Shiojima et al., 1999; Morin et al., 2000; Suparna et al., 2001; Dai et al., 2002; Habets et al., 2002; Thattaliyath et al., 2002], suggest that synergistic action between the two transcriptional factors fine-tune gene expression and heart development. For example, dHAND physically interacts with transcription factors MEF2C, GATA4, and

NKX2.5, which are co-expressed together in the heart, all these combinatorial interaction results in synergistic activation of the ANP promoter. Our data suggest that MEF2C protein physically interact with dHAND and recruit dHAND. Similarly, MEF2C is recruited by GATA4 to the ANP promoter to potentiate transcription, so if dHAND, MEF2C, and GATA4 together regulate the expression of ANP on the same temporal and spatial, it is impossible that GATA4 substitute for dHAND at the MEF2C site on the ANP promoter. On the other hand, dHAND physically interact with NKX2.5 and GATA4, it is also recruited by NKX2.5 to the ANP promoter, but the interaction between dHAND and GATA4 is dependent upon P300, which is classified as the transcriptional coactivator. These data place dHAND center stage with respect to interactions with GATA4, NKX2.5, and MEF2C, and this also suggests that the protein–protein interactions between these cardiac-restricted transcription factors and protein-DNA interaction contributed to assembly of higher-order cardiac-specific enhanceosome, which is aligned at precise spatiotemporal manner and exert a specific biological response.

Role of ANP in Regulating Heart Development

During heart development, a panel of cardiac specific genes, such as ANP, brain naturetic peptide (BNP), cardiac-actin, and myosin heavy chain, play important roles in contractile, conduction, and metabolic regulation. ANP, being a kind of cardiac specific gene, play an important role in the regulation of cardiovascular and renal homeostasis. Atrial naturetic peptide possesses significant diuretic, natriuretic, and vasodilatory activity. It plays a critical roles in the maintenance of blood pressure and sodium balance in both normal and hypertensive states. Expression of ANP is developmentally regulated in the mammalian heart. During embryonic development, the ANP gene is expressed in both the atrium and the ventricle, shortly after birth its expression is downregulated in the ventricle, leaving the atrium as the predominant site of ANP biosynthesis and storage [Wu et al., 1988]. However, when ventricles are subjected to various stimuli, including hormones, genetic propensity, hypertension, pressure and hemodynamic overload, expression of the ventricular ANP gene is reactivated. Ventricular reexpression of ANP during hypertrophic growth may thus contribute to circulating levels of the hormone and play a potentially important role in hemodynamic regulation during hypertension. This induction of the ANP gene is a highly conserved feature of ventricular hypertrophy in all mammalian species including mouse, rat, hamster, dog, and humans. So ANP not only play important roles in heart development but also in ventricular hypertrophy. Accordingly, ANP is a good model for studying mechanisms that regulate cardiac-specific gene expression, which made us to better understand the molecular mechanisms involved in the normal cardiac development and cardiac hypertrophy.

Our study demonstrates that dHAND and MEF2C form protein complex through interaction to cooperatively activate the ANP promoter. So they both act as players in the regulation of cardiovascular homeostasis. Finally, we will examine whether several signaling cascades including epithelial-1 and p38 MAP kinase influence the dHAND-MEF2C synergy on the ANP promoter [Finn et al., 2001]. Based on the previous reports, P38 MAPK activate MEF2 in cardiac muscle [Kato et al., 2000; Finn et al., 2001], dHAND also has been implicated in epithelial-1-induced transcription [Thomas et al., 1998; Han and Molkentin, 2000], so it will be interesting to test whether integrating multiple signaling pathways regulate the dHAND-MEF2C physical interaction and functional synergy on ANP to maintain blood volume homeostasis by rapid and subtle alterations of ANP expression.

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