Regulation of the HMOX1 gene by the transcription factor AP-2δ with unique DNA binding site

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Abstract. AP-2 transcription factors are important sequence-specific DNA-binding regulators that are expressed in the neural crest and other tissues during mammalian development. The human AP-2 family of transcription factors consists of five members, AP-2α, -β, -γ, -δ and -ε, which have an important role in the regulation of gene expression during development and in the differentiation of multiple organs and tissues. The present study aimed to investigate the mechanism by which AP-2δ mediates heme oxygenase-1 (HMOX1) gene expression. It was identified that the human AP-2δ protein exhibited weak binding to a suboptimal AP-2 sequence, 5'-GCCN3GGC-3', to which all other AP-2 proteins bind *in vitro*, providing the first example of DNA target specificity amongst the AP-2 family. AP-2δ protein bound to an optimized AP-2 consensus DNA sequence, 5'-GCCTGAGGC-3', *in vitro* and transactivated gene expression in eukaryotic cells. The transactivation domain of Ap-2δ differs notably from those in the other AP-2 proteins as it lacks the PY motif (XPPXY) and several other conserved residues that are important for the transcriptional activity of AP-2 proteins, yet it functions as an equally strong activator.

Introduction

Members of the AP-2 family of transcription factors have important roles in several cellular processes, including apoptosis, migration and differentiation. Furthermore, AP-2 transcription factors have been implicated in carcinogenesis, a process where the normal program of cell growth and differentiation is disrupted. The human AP-2 family of transcription factors consists of five members, AP-2α, -β, -γ, -δ and -ε. The C-terminal half of the AP-2 proteins contains a basic domain and a helix-span-helix motif that mediates DNA binding and dimerization. This portion of the AP-2 protein is highly conserved among all AP-2 family members. The N-terminal half of AP-2 proteins contains the transactivation domain. The transactivation domains of AP-2 proteins are significantly more divergent, although certain critical residues and a PY motif (XPPXY) are conserved (1-6). A number of functional AP-2 binding sites, consensus to a palindromic core sequence, 5'-GCCN$_3$GGC-3', have been identified in cellular and viral enhancers, and preferred binding to the sequence motifs GCCN$_3$GGC, GCCN$_4$GGC and GCCN$_{3/4}$GGG was observed in an *in vitro* binding site selection assay (7,8). The PY motif and critical residues in the transactivation domain, which are considered necessary for transactivation, were divergent in Ap-2δ. The unique protein sequence and functional features of Ap-2δ implicate other underlying mechanisms, besides tissue-specific AP-2 gene expression, for the specific control of target gene activation.

Despite the considerable sequence similarities and overlap in the expression of AP-2 family members, it has been described that each of the five AP-2 genes exhibits a distinct expression pattern in mouse embryos, suggesting that each of them may have a different role in development (9). In addition, the knockout of individual AP-2 members in mice results in specific developmental defects (10,11). Consequently, identification of the specific targets of each member is highly important in further understanding the function of AP-2.

Heme oxygenase-1 (HMOX1) has emerged as a key cytoprotective gene and enzyme in numerous experimental and clinical contexts. The HMOX1 gene is under complex regulation and is markedly upregulated by heme, the physiological substrate for the HMOX1 protein, by iron and other transition metallic ions, and by oxidative and heat stress and other stressful perturbations (12-14). Regulation of the HMOX1 gene expression is associated in part with alterations in the levels of several transcription factors, including Bach1 and nuclear factor-erythroid 2-related factor 2 (Nrf2) (15-18). Ap-2δ was identified as a new activating transcription factor of HMOX1. However, the mechanism underlying the regulation of HMOX1 expression by Ap-2δ remained to be elucidated. The present study aimed to investigate the mechanism by which AP-2δ mediates HMOX1 gene expression.

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Materials and methods

Plasmid constructs. cDNA encoding human AP-2δ (GenBank Accession Number AY028376) was generated by PCR from human brain cDNA library (Invitrogen Life Technologies, Carlsbad, CA, USA). The coding region of AP-2δ cDNA was amplified by PCR using primers (5'-GGGGTACCATG TCAACTACCTTTCCGGGAC-3' and 5'-CCCTCGAGGGT CTGTCTTTTCTGTTTTGCCCTC-3') containing *Hin*dIII and *Eco*RI restriction sites and then it was subcloned into the vector pcDNA4C (maintained in our laboratory) to generate the construct pcDNA4C-AP-2δ. The primers were obtained from Biomics Biotechnologies (Nantong, China). The constructs were verified by DNA sequencing (Biomics Biotechnologies).

Cell culture and transient transfection. AD293 cells and NIH3T3 cells obtained from Shanghai Institute of Cell Biology and Biochemistry (Shanghai, China), were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA). The cells were split on 60 mm dishes at $1x10^6$/dish. Following 24 h, the cells were transfected using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Each 60 mm dish was transfected with 10 μg of pcDNA4C-AP-2δ. An empty vector, pcDNA4C, was used as a negative control for the transfection assays.

Luciferase activity assay. The pGL3-basic-HMOXI reporter constructs containing the 1360 bp upstream sequences of the human HMOXI gene (GenBank Accession Number BC001491), were inserted into the pGL3-basic-vector (Promega Corporation, Madison, WI, USA). To generate HMOX1-1360luc plasmids, PCR was performed using the following oligonucleotides: Sense primer, 5'-CCGCTC GAGGAATACAGTAGCGTGGTCAC-3' and antisense primer, 5'-CCGCTCGAGATGCCAGGCCTGAAAGC CAT-3'. The 1,100 bp (sense primer: 5'-CCGCTCGAGATGCC AGGCCTGAAAGCCAT-3'),850 bp (sense primer: 5'-CCGCTCGAGAGAGTGTCCCACGCATTCCA-3'), 637 bp (sense primer: 5'-CCGCTCGAGCAGGTCAGTTGTAGGGA TGAACC-3'), 460 bp (sense primer: 5'-CCGCTCGAGGTTC CTGATGTTGCCCACCA-3'), 206 bp (sense primer: 5'-CCGCTCGAGCTATGGCCAGACTTTGTTTCC-3') and 113 bp (sense primer: 5'-CCCAAGCTTTCGAGAGGAGGCA GGCGTT-3') fragments were isolated following digestion of the PCR products with *Xho I* and *HindIII*, and were subcloned to the pGL3-basic vector, resulting in HMOX1-1100luc, HMOX1-850luc, HMOX637luc, HMOX1-460luc, HMOX1-206luc and HMOX1-113luc plasmids, respectively. The same anti-sense primer (5'-CCCAAGCTTTCGAGAG GAGGCAGGCGTT-3') was used for all deletional mutant luciferase reporters. The primers were obtained from Biomics Biotechnologies. The upstream and junction regions of these constructs were confirmed by sequence analysis (Biomics Biotechnologies).

Base substitutions at the AP-2 binding sites were generated in the context of HMOX1-206luc and HMOX1-113luc using the Transformer™ Site-Directed Mutagenesis kit (Clontech Laboratories, Inc., Mountain View, CA, USA). HMOX1-206luc^m mutated from 5'-ccgccccgggccagtgtg-3' to 5'-ccgccccgaatcagtgtg-3' and HMOX1-113luc^m mutated from 5'-cagctgttccgcctggccca-3' to 5'-cagctgttattcctggccca-3' (underlined bases represent mutated sequences) according to the manufacturer's instructions. Constructs with correct mutations were confirmed by direct sequence analysis (Biomics Biotechnologies).

A total of 24 h following transfection, the cells were seeded into 96-well plates ($8x10^3$ viable cells/well) and allowed to attach overnight. A total of 200 ng of pGL3-HMOX1-5' untranslated region (UTR) or pGL3-mut HMOX1-5'UTR plus 80 ng pRL-SV40 (Promega Corporation) were transfected in combination with pcDNA4C-AP-2δ or empty vector pcDNA4C (final concentration, 80 nM) using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Luciferase activity was measured 48 h following transfection using the Dual Luciferase Reporter Assay system (Promega Corporation). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. Three independent experiments were performed in triplicate.

Electromobility shift assays (EMSA). Double-stranded AP-2 oligonucleotides end-labeled with biotin were obtained from Promega Corporation. The biotin-labled sequences were as follows: HMOX1-1: 5'-AGTTCCTGATGTTGCCCACCAGG CTATTGCTCTGAGCAGC-3', HMOX1-2: 5'-CTCCTCTCC ACCCCACACTGGCCCGGGGCGGGCTGGGCGC-3'. The optimized biotin-labled AP-2 binding sequence was as follows: 5'-GAACTGACCGCCTGAGGCGCGTGTGCAGAG-3'. The four overlapping and biotin-labeled sections were as follows: HMOX1-3 (-206 to -149 bp of HMOX1 5'UTR), HMOX1-4 (-161 to -104 bp of HMOX1 5'UTR), HMOX1-5 (-113 to -56 bp of HMOX1 5'UTR) and HMOX1-6 (-65 to -1 bp of HMOX1 5'UTR), respectively. Binding was performed at room temperature in a reaction mixture that included 2 μl of Ap-2δ protein directly from nuclear extracts, 20 fmol labeled AP-2 oligonucleotide, 4% glycerol, 1.0 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris (pH 7.5) and 0.05 mg/ml poly(deoxyinosinic-deoxycytidylic) acid. The products were fractionated on 4% non-denaturing polyacrylamide gels at room temperature. Binding specificity was determined using 200-fold concentrations of unlabeled AP-2 oligonucleotide during the binding reactions. To characterize the specificity of the DNA binding of Ap-2δ, competition assays were performed by competing a biotin-labeled optimized AP-2 oligonucleotide. For comparison, the human AP-2α protein was translated *in vitro* and then subjected to competition assays with the same ratios of optimized AP-2 oligonucleotides. All of the experiments were repeated three times.

Results

Exogenous expression of AP-2 robustly transactivates HMOX1 activities in AD 293 and NIH3T3 cell lines. Through bioinformatics analysis, it was identified that the 5' proximal promoter regions of HMOX1 genes contain two consensus AP-2 motifs. To examine whether exogenous expression of AP-2δ activates HMOX1 promoter activity, AP-2δ expression vector was

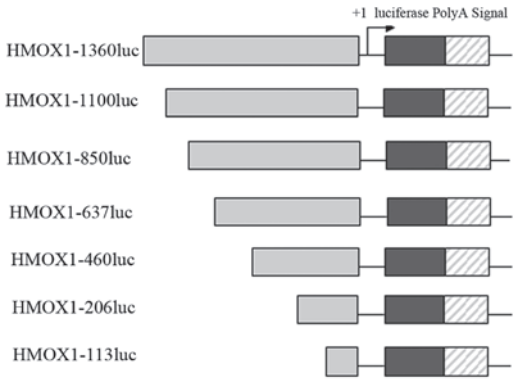

Figure 1. Deletional analysis of the HMOX1 promoter for its transactivation by AP-2. Several deletional mutants of HMOX1-luc reporter plasmids were generated and co-transfected with pcDNA4C-AP-2 or pcDNA4C into the AD293 or NIH3T3 cell lines. HMOX1, heme oxygenase-1; luc, luciferase.

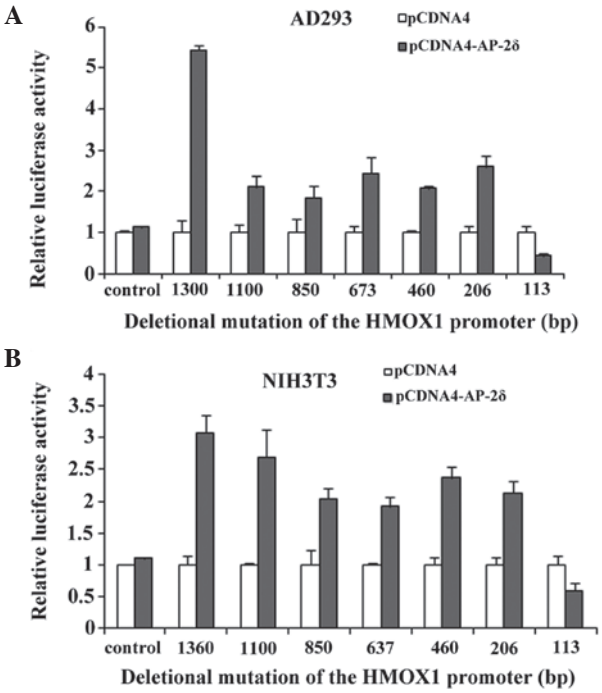

Figure 2. Differential effects of deletional mutation of the AP-2 binding sites of the HMOX1 promoter. The effects on basal as well as AP-2-inducible transcriptional activities were examined in (A) AD293 and (B) NIH3T3 cell lines. The cells were harvested 48±72 h following transfection. Luciferase activity was determined and normalized to the activity of β-galactosidase . HMOX1, heme oxygenase-1.

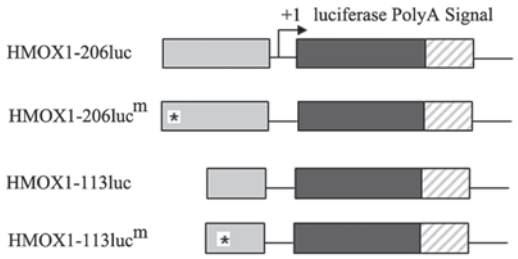

Figure 3. Base substitutions at the AP-2 binding sites were generated in the context of HMOX1-206luc and HMOX1-113luc. HMOX1-206luc^m mutated from 5'-ccgccccgggccagtgtg-3' to 5'-ccgccccgaatcagtgtg-3' and HMOX1-113luc^m mutated from 5'-cagctgttccgcctggccca-3' to 5'-cagctgttattcctggccca-3' (underlined bases represent mutated sequences). *Mutated sequence. HMOX1, heme oxygenase-1; luc, luciferase.

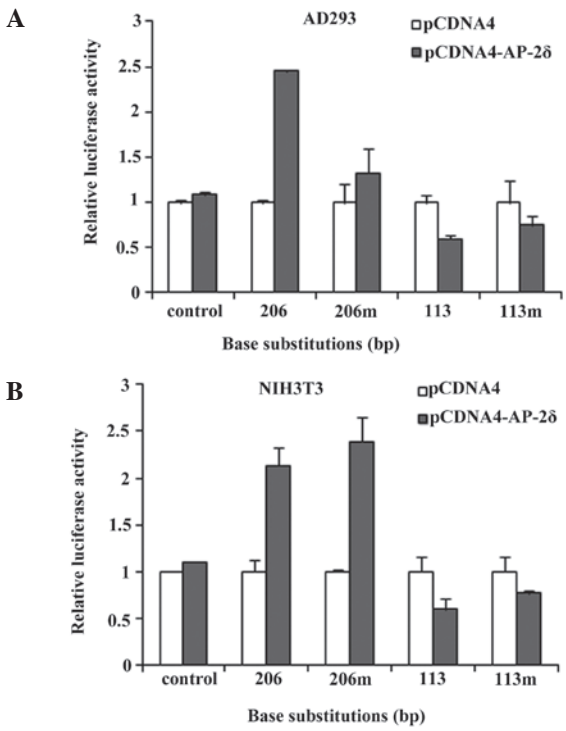

Figure 4. Differential effects of mutation of the AP-2 binding sites of the HMOX1 promoter. Base substitutions were introduced as demonstrated in Fig. 3. Their effects on basal as well as AP-2-inducible transcriptional activities were examined in (A) AD293 and (B) NIH3T3 cell lines. Cells were harvested 48±72 h following transfection. Luciferase activity was determined and normalized to the activity of the β-galactosidase. HMOX1, heme oxygenase-1.

co-transfected with the reporter construct HMOX1-1360luc to AP-2δ-positive (AD293) and AP-2δ-negative (NIH3T3) cell lines.

To obtain further direct evidence that the HMOX1 gene is a target of AP-2δ, the binding site of AP-2δ in the 5'UTR of HMOX1 was investigated. Several deletional mutant luciferase reporters were constructed (HMOX1-1100luc, HMOX1-850luc, HMOX-637luc, HMOX1-460luc, HMOX1-206luc and HMOX1-113luc; Fig. 1). Correspondingly, two mutant reporters (HMOX1-206luc^m and HMOX1-113luc^m) were also generated, in which the sequences in AP-2 consensus binding sites were changed (Fig. 3). Each of these constructs were co-transfected with PcDNA4C-AP-2α and pcDNA4C-AP-2δ plasmid into the AD293 and NIH3T3 cells. An empty vector, pcDNA4C, was used as a negative control for the tranfection assays.

The assays demonstrated that the luciferase activity in the HMOX1-1360luc-transfected cells was significantly increased by three-fold compared with the luciferase activity in the negative control cells, suggesting that AP-2δ increased the luciferase activity of HMOX1 in the two cell lines (Fig. 2). Therefore, it was concluded that the inserted fragment of HMOX1 (position -1 to -1360 bp of HMOX1 5'UTR) was the target of AP-2δ.

To examine which fragment was the regulatory sequence, the various HMOX1-luc constructs containing different lengths of HMOX1 upstream sequences for their transcriptional activation in response to exogenous AP-2 expression

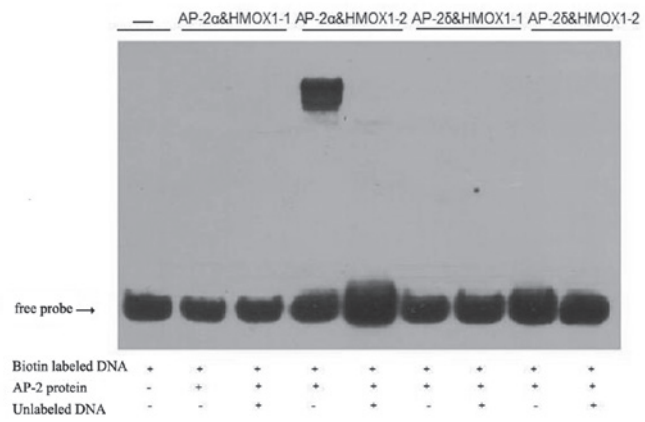

Figure 5. Specific DNA/protein interaction between AP-2 motifs identified in the HMOX1 upstream region (HMOX1-1 and HMOX1-2) and the recombinant AP-2 protein. Biotin-labeled oligonucleotides encompassing the individual AP-2 binding motifs were used as probes in EMSA and were incubated with the recombinant AP-2 protein. Competition assays were performed with 200-fold concentrations of unlabeled AP-2 oligonucleotides. Free probe is indicated by the arrow. HMOX1, heme oxygenase-1; EMSA, electromobility shift assay.

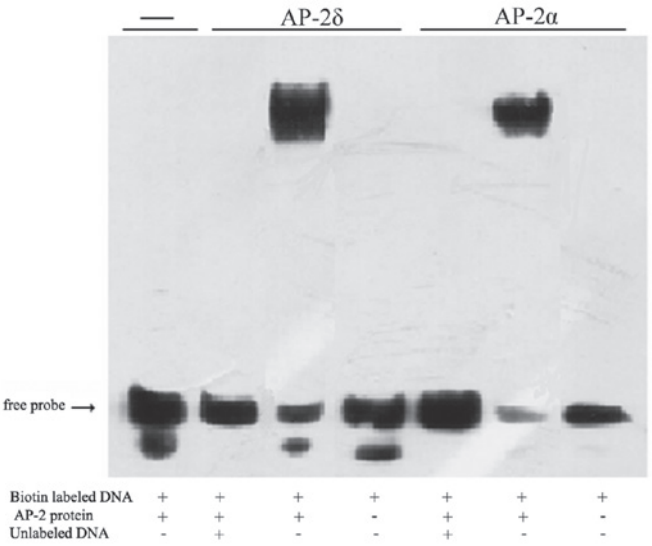

Figure 6. Specific DNA/protein interaction between optimized AP-2 binding sequence and the recombinant AP-2 protein. Biotin-labeled oligonucleotides encompassing the individual AP-2 binding motifs were used as probes in EMSA and were incubated with the recombinant AP-2 protein. Competition assays were performed with 200-fold concentrations of unlabeled optimized AP-2 binding sequence. Free probe is indicated by the arrow. HMOX1, heme oxygenase-1.

were investigated (Fig. 1). Deletions starting from the -1360 kb to the -206 kb site did not change the AP-2-responsive promoter activation of the HMOX1 gene, which was activated more than once, both in the AD293 and NIH3T3 cell lines. However, transcriptional activation of HMOX1-113luc in response to exogenous AP-2 expression was diminished by 1-fold, evidently suggesting that the HMOX1 promoter from -206 to -1 may contain multiple AP-2-responsive sites.

Furthermore, two mutant reporters (HMOX1-206luc^{m} and HMOX1-113luc^{m}) were generated, in which the sequences in AP-2 consensus binding sites were changed (Fig. 3). The AP-2δ responsiveness of the HMOX1 promoter was affected only

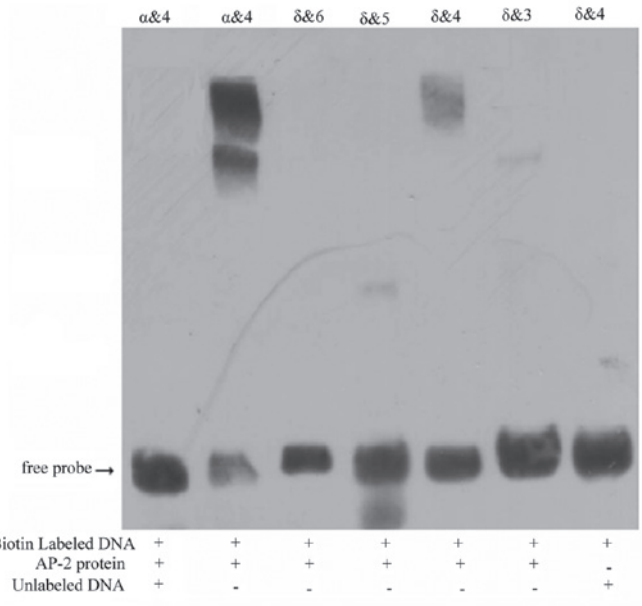

Figure 7. Specific DNA/protein interaction between four overlapping sections of HMOX1 promoter from -206 to -1 and the recombinant AP-2 protein. The four sections were HMOX1-3 (-206 to -149 bp), HMOX1-4 (-161 to -104 bp), HMOX1-5 (-113 to -56 bp) and HMOX1-6 (-65 to -1bp), respectively. Competition assays were performed with 200-fold concentrations of unlabeled optimized AP-2 binding sequence. Free probe is indicated by the arrow. HMOX1, heme oxygenase-1.

marginally, if at all, by this mutation in the cell lines (Fig. 4). These data led to the hypothesis that transcriptional activation of the HMOX1 gene by AP-2δ may require additional cis-elements, rather than traditional AP-2 consensus binding sites.

Sequence-specific DNA binding. Ap-2 proteins have been reported to bind to a highly conserved GC-rich consensus sequence present in numerous cellular and viral promoters and enhancers. An *in vitro* binding site selection assay identified GCCN3/4GGC and GCCN3/4GGG as the preferred sequence motifs bound by Ap-2 transcription factors (7,8). Two multiple protein-binding sites were identified in the HMOX1 upstream promoter regions, which were HMOX1-1, 5'-agttcctgatgttgcccaccaggctattgctctgagcagc-3' (underlined bases represent similar AP-2-binding sequence) and HMOX1-2, 5'-ctcctctccacccca-cactggcccggggcgggctgggcgc-3' (underlined bases represent consensus AP-2-binding sequence). Domain HMOX1-2 of the HMOX1 promoter includes a consensus AP-2-binding motif (5'-GCCN3GGC-3'). Domain HMOX1-1 includes a similar AP-2-binding sequence (5'-GCCN5GGC-3'). Initially, empty pcDNA4C, Ap-2δ and AP-2α genes were translated *in vitro*, producing equivalent amounts of protein, and then gel shift assays were performed using the AP-2 binding sequence derived from the HMOX1 upstream promoter. These studies revealed consistently shifted bands with domain HMOX1-2 when the AP-2α protein was used, but weak and inconsistent shifts were observed when Ap-2δ was employed (Fig. 5).

Previous studies have demonstrated the importance of the flanking sequence of the core 5'-GCCNNNGCC-3' sequence. It was identified that the presence of a guanosine at the -1 position on either DNA strand significantly reduced AP-2 binding. In addition, the study reported that TGA was the preferred

sequence for the central 3 bp between the palindromic GCC and GGC motifs (19). The two HMOX1 AP-2 binding sequences did not conform to those rules, so the gel shifts were repeated using an oligonucleotide with an optimized sequence (5'-GAACTGACCGCCTGAGGCGCGTGTGCAGAG-3'). Under these conditions, Ap-2δ protein induced a strong and consistently shifted band. To document DNA binding specificity, 200-fold amounts of unlabeled optimized AP-2 oligonucleotide were added in competition. Cold AP-2 oligonucleotide significantly reduced the intensity of the shifted band comprised of the biotin-labeled AP-2 oligonucleotide/Ap-2δ protein complex (Fig. 6). These results showed that Ap-2δ protein binds to the optimized AP-2 consensus sequence with specificity. In addition to the HMOX1 results, it appeared that Ap-2δ exhibited a higher DNA sequence preference than AP-2α under *in vitro* conditions.

The luciferase assays demonstrated that the HMOX1 promoter from -206 to -1 may contain multiple AP-2-responsive sites. It was observed that the sequence from -206 to -1 did not contain the optimized sequence 5'-GCCTGAGCC-3', which indicates the existence of other AP binding sites. To examine the binding sites, the 206 bp were divided into four overlapping sections and biotin-labeled. The four sections were HMOX1-3 (-206bp to -149bp), HMOX1-4 (-161bp to -104bp), HMOX1-5 (-113bp to -56bp) and HMOX1-6 (-65bp to -1bp), respectively. For comparison, an empty vector, pcDNA4C, was used as a negative control and human AP-2α protein was translated *in vitro* as a positive control. From Fig. 7, HMOX1-3, HMOX1-5 and HMOX1-6 did not generate any detectable complexes with the recombinant AP-2δ protein. AP-2δ protein induced a consistently shifted band with HMOX1-4. A total of 200-fold amounts of unlabeled optimized AP-2 oligonucleotide significantly reduced the intensity of the shifted band, comprised of the biotin-labeled AP-2 oligonucleotide/AP-2δ protein complex.

Discussion

The AP-2 family members define a distinct class of transcription factors characterized by a highly conserved C-terminal basic region and helix-span-helix motif, which are critical for DNA binding functions (20). In the present study, it was identified that AP-2 transcription factors have a close to identical binding specificity (21). AP-2δ, however, binds weakly to the conserved GCCN3/4GGC sequence *in vitro*, providing the first example of target sequence specificity among the AP-2 family. It was identified that the AP-2δ protein binds to an optimized AP-2 consensus sequence with specificity. In addition to the HMOX1 results, it also appeared that AP-2δ exhibited a higher DNA sequence preference than AP-2α under *in vitro* conditions. Based on these results, AP-2δ was able to transactivate luciferase expression in cell culture using the HMOXI promoter which did not contain the optimized AP-2-binding oligonucleotide. This was expected, as it has been previously demonstrated that upon overexpression in cell culture, AP-2 proteins perform transactivation using DNA sequences for which they have relatively weak affinity (22). The binding specificity of AP-2 proteins is considered to reside within the basic region (23). Therefore, it is noteworthy that four residues in that domain, which are completely conserved among all other AP-2 proteins, differ in AP-2δ (Asp209, Leu210, Lys235 and Ile252). These substitutions, often conservative, may provide clues about contact points between AP-2 with its DNA binding sequence.

By contrast, N-terminal sequences of the AP-2 transactivation domains are weakly conserved compared with the basic and helix-spanhelix regions (23). This sequence divergence is hypothesized to result in the varying transactivation efficiency observed among the AP-2 family members, presumably through altered co-activator interactions. Certain residues in the transactivation domain are highly conserved, and alterations in these amino acids often have marked adverse effects on transactivation (24,25). These residues are highly conserved in the human AP-2 proteins; however, they are present as Thr86 and Ser91 in AP-2δ. The effectiveness of AP-2δ in transactivation, despite those evolutionary substitutions, suggests that AP-2δ may form complexes with a substantially different set of co-activators than the other AP-2 proteins. This provides another level of control for the induction or repression of gene transcription.

Although the five human AP-2 proteins are highly similar in their C-terminal halves, they appear to vary in function during development. This diversity, despite their structural similarity, may arise in various ways. Homologous AP-2 genes are expressed in spatially and temporally distinct patterns (26,27). In one study, PCR analysis demonstrated that AP-2δ was expressed at high levels in adult thymus, prostate, small intestine, skeletal muscle, placenta, brain and testis tissues. Subsequent studies demonstrated that AP-2δ may significantly regulate certain genes, including RPL29, CAPNS1, HMOX1, PDGFC and RPL29 (28).

In the present study, the regulatory function of AP-2δ on HMOX1 expression was examined. HMOX1 is a multitask enzyme that has an important role in the regulation of cell proliferation, differentiation, oxidative status and apoptosis, thereby affecting immune responses, inflammatory reaction and angiogenesis (13,14). Therefore, its significance is notably wider than only haem elimination. HMOX1 is one of the most highly induced genes in cells exposed to stressful conditions, and hundreds of experiments have demonstrated that the products of HMOX1 activity are involved in the maintenance of cell homoeostasis (29). The importance of HMOX1 in cardiovascular diseases and cancer progression has been commonly accepted, particularly when considering the variability of HMOX1 in the human population, resulting from HMOX1 promoter polymorphism (30). Regulation of HMOX1 gene expression is associated in part with alterations in the levels of several transcription factors. In the present study, Ap-2δ was identified as a new activating transcription factor of HMOX1. Further investigations will focus on examining the regulatory function of AP-2δ on HMOX1 *in vivo*. Future studies are expected to provide more data elucidating the complex interactions of HMOX1 within this elaborate gene-regulatory system.

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