JunB and JunD Regulate Human Heme Oxygenase-1 Gene Expression in Renal Epithelial Cells*

Received for publication, September 1, 2006, and in revised form, November 24, 2006. Published, JBC Papers in Press, January 3, 2007, DOI 10.1074/jbc.M608456200

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Heme oxygenase-1 is a highly inducible gene, the product of which catalyzes breakdown of the prooxidant heme. The purpose of this study was to investigate the regulation of the human heme oxygenase-1 gene in renal epithelial cells. DNase I hypersensitivity studies identified three distal sites (HS-2, -3, and -4) corresponding to approximately −4.0, −7.2, and −9.2 kb, respectively, of the heme oxygenase-1 promoter in addition to one proximal region, HS-1, which we have shown previously to be an E box. In vivo dimethyl sulfate footprinting of the HS-2 region revealed six individual protected guanines. Two mutations within HS-2 combined with a third mutation of the proximal E box abolished heme- and cadmium-driven heme oxygenase-1 promoter activation, suggesting that these three sites synergized for maximal heme oxygenase-1 induction. Jun proteins bound to the antioxidant response element in the HS-2 region in vitro and associated with the heme oxygenase-1 promoter in vivo. JunB and JunD contribute opposing effects; JunB activated whereas JunD repressed heme oxygenase-1 expression in human renal epithelial cells, results that were corroborated in junB−/− and junD−/− cells. We propose that heme oxygenase-1 induction is controlled by a dynamic interplay of regulatory proteins, and we provide new insights into the molecular control of the human heme oxygenase-1 gene.

Heme oxygenase-1 (HO-1)3 catalyzes heme degradation resulting in the release of equimolar amounts of biliverdin, iron, and carbon monoxide (1, 2). Biliverdin is subsequently reduced to bilirubin via biliverdin reductase. Two isoforms of HO have been described as follows: a highly inducible form, HO-1, and a constitutive isoform, HO-2. HO-1 is induced by several stimuli such as heme, heavy metals, cytokines, growth factors, nitric oxide, oxidized lipids, hydrogen peroxide, shear stress, glucose deprivation, angiotensin II, statins, as well as others (3). Recent reports have demonstrated the critical importance of HO-1 expression in mediating anti-oxidant, anti-inflammatory, and anti-apoptotic effects in several disease states, including acute renal failure, organ transplant rejection, atherosclerosis, sepsis, ischemia-reperfusion injury, and vascular restenosis (2, 4). These have been confirmed both in vitro and in vivo using pharmacological as well as genetic approaches to modulate HO-1 expression in animal models. The importance of HO-1 has also been corroborated in Ho-1 knock-out mice and a patient with HO-1 deficiency, both exhibiting increased susceptibility to inflammatory injury (5, 6).

The mechanisms underlying HO-1 induction by multiple inducers are regulated predominantly at the level of transcription. Previous studies pertaining to the regulation of the mouse Ho-1 gene have focused on the transcription factors, NF-E2-related factor 2 (Nrf2) and Bach1 (7, 8). Nrf2 has been associated with the induction of phase 2 detoxifying enzymes that include but are not limited to NAD(P)H quinone oxidoreductase, glutathione S-transferases, HO-1, aldo-keto reductases, ferritin, and UDP-glucuronosyltransferases (reviewed in Ref. 9). These genes contain an extended AP-1-like element in their promoters that has been referred to as a stress-response element (SRE) or an antioxidant-response element (ARE) (10, 11). Upon exposure to stress, Nrf2 is proposed to be released from Keap1, its cytosolic binding partner, which then translocates to the nucleus and binds to the promoters of genes resulting in transcriptional activation (12–16). Recent studies, however, have challenged this concept and have suggested that Nrf2 is mostly nuclear and is recruited to the DNA constitutively to activate gene expression (17). On the other hand, Bach1, a bZip transcription factor, has been identified as a negative regulator of mouse Ho-1 induction (18). When stimulated with heme, the heme binds to Bach1 (8, 18), releasing Bach1 from the ARE motif, allowing Nrf2 to bind to the ARE and activate transcription (8, 18).

Previous studies have reported the presence of both positive and negative regulatory sequences in the human HO-1 promoter (19). Consensus sequences for DNA-binding proteins,
such as nuclear factor-κB, activator protein-1 (AP-1), AP-2, Sp1, upstream stimulatory factor (USF), c-Myc/Max, and inter-leukin-6 response elements as well as other transcription factors, have been reported in the promoter region of the human HO-1 gene (20–25). Most of these results were derived from promoter deletion studies, in vitro DNase I footprinting, or computer-based consensus sequence predictions. The ARE core sequence, 5′-TGCTGAGTCA-3′ (26), is present in both the mouse and human HO-1 promoters. Within this sequence is a classical AP-1 site (TGAGTCA) that is a binding site for the Jun family of proteins, c-Jun, JunB, and JunD (27–32). Juns dimerize with themselves or with other members of the AP-1 family (e.g. c-Fos, FosB, Fra1, and Fra2) (33). The downstream effects of Jun proteins have only recently been unveiled (reviewed in Refs. 34 and 35). For example, mice deficient in JunB die early in embryogenesis due to lack of proper placenta formation (36). JunD overexpression has been shown to reduce tumor angiogenesis (37), whereas lack of JunD promotes angiogenesis in the heart (38, 39).

In the work presented here, using in vivo footprinting and DNase I hypersensitivity analyses of the human HO-1 promoter, we show that three regions containing a cAMP-response/AP-1 element (CRE/AP-1), ARE, and E box, respectively, are cooperatively required for hemin and cadmium-mediated HO-1 promoter activation in human renal epithelial cells. We provide evidence that JunB and JunD associate with the HO-1 promoter in vivo and are capable of influencing HO-1 expression in an opposing manner.

**EXPERIMENTAL PROCEDURES**

Reagents—Tissue culture media, serum, and supplements were obtained from Invitrogen. Hemin, cadmium chloride, and dimethyl sulfate (DMS) were purchased from Sigma. DNase I was from Worthington. Restriction endonucleases and reagents for PCR, including synthetic oligonucleotides, were from New England Biolabs (Beverly, MA) and Invitrogen, respectively. Anti-JunD (sc-74), anti-pan-Jun (sc-44), anti-c-Jun (sc-1694), anti-JunB (sc-8051), anti-Nrf2 (sc-722 and sc-13032), anti-Nrf1 (sc-721), and anti-USF1 (sc-229) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-menin antibody (A300-105A) was from Bethyl Laboratories (Montgomery, TX); anti-HO-1 (SPA-895) was from StressGen, and anti-V5 (R960-25) antibody was from Agene, La Jolla, CA) according to the manufacturer’s instructions. The JunB expression plasmid was constructed by amplifying the JunB cDNA from HK-2 cell-derived cDNA using the primers 5′-atgtgcactaaaatggaacagcccttc-3′ (forward) and 5′-gaaggcgtgctcccttgaccccaagtcctcctc-3′ (reverse), 5:1 units/unit mixture of Taq and Vent DNA polymerase, and subsequently cloned into pcDNA3.1/V5-His Topo vector (Invitrogen). The clone was sequence-verified. The JunD expression plasmid constructs were kindly provided by Dr. Jean-Michel Mesnard (47). The c-Jun expression plasmid was from Dr. Rik Derynck (University of California, San Francisco).

HK-2 cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions using a batch transfection protocol as described previously (46). Briefly, the indicated HO-1 promoter construct was transfected into HK-2 cells at ~90% confluency in a 10-cm tissue culture dish. Twenty four hours post-transfection the cells were split into two or three 10-cm dishes (for hGH mRNA analyses) or into a 12-well tray (for luciferase assays), in order to ensure equal transfection efficiency between experimental treatments. After the cells were allowed to recover for 16 h, each dish was treated with the stimulus (hemin or cadmium) or vehicle (control) and assayed for hGH mRNA by Northern analysis. For luciferase assays, 6 of the 12 wells derived from transfection of the promoter construct in the 10-cm dish were treated with vehicle (control) or stimulus and assayed for luciferase activity. In control experiments, wherein pcDNA3.1/lacz was co-transfected using the batch transfection protocol, equal amounts of β-galactosidase expression were observed in each of the wells (46). For T47D transfections, cells were plated in 24-well plates and transfected with FuGENE 6 (Roche Applied Science) and pHOGL3/4.5 or mutated constructs (150 ng/well), a JunB or JunD expression plasmid (150 ng/well), and pCMX-β-gal (50 ng/well). Twenty four hours after transfection, cells were treated with control media or media containing hemin (5 μM). After an additional 24 h, cells were lysed, and luciferase activity was measured and normalized to β-galactosidase activity for each well.
DNase I Hypersensitivity—DNase I hypersensitivity assays were performed as described previously (48). Briefly, HK-2 cells were treated with hemin (5 μM) or vehicle (Me₅SO) for 2 h. Cells were collected and nuclei isolated. The nuclei were exposed to increasing amounts of DNase I for 45 s at 37 °C. The nuclei were then lysed in a standard SDS/proteinase K buffer. DNA was purified using the salt out technique (adding 1/3 volume of saturated NaCl to precipitate the SDS) followed by ethanol precipitation. The DNA was then digested with EcoRI or HindIII. 15 μg of digested DNA was electrophoresed, blotted, and hybridized with a random prime (Invitrogen)-labeled 225-bp probe that abuts the EcoRI site at −1.4 kb and extends distally.

In Vivo DMS Footprinting and LMPCR—HK-2 cells were grown to ~80% confluency on 15-cm collagen-coated plates and treated with hemin (5 μM), cadmium (10 μM), or Me₅SO for 2 h. Cells were washed once at room temperature with PBS followed by DMS (0.5% in PBS) treatment for 30 s with gentle agitation. In vivo footprinting and ligation-mediated PCR (LMPCR) were performed as described previously (21, 49).

After DMS treatment and pipendine cleavage, the oligonucleotide 5'-GAGGAGAATATCCAGGCAAG-3' was annealed and extended with Vent DNA polymerase (New England Biolabs). The linker was ligated to the DNA fragments and subsequently amplified for 25 cycles using the HO-1 nested primer 5'-GCTGCCCCAAAACACTTCTGTTCCTGTA-3' and the linker primer 5'-GCCGGTGACCCGGGATCTGAATTC-3' (Table 1). Amplicons were electrophoresed on a 5% acrylamide, 7% urea sequencing gel, electroblotted, and hybridized at 45 °C with the EcoRI site at −1.4 kb and extends distally.

Northern Analysis—Following transfections, Northern analysis was performed as described previously (21). HK-2 cells grown on 10-cm collagen-coated dishes were transfected with the indicated HO-1 reporter constructs (4 μg) and, where indicated, co-transfected with an equimolar ratio of c-Jun, JunB, or JunD. Each dish was then split into two or three 10-cm dishes. After a 16-h recovery, cells were cultured with hemin (5 μM), cadmium chloride (10 μM), or vehicle (Me₅SO) for 16 h. Total RNA was extracted, electrophoresed, blotted, and sequentially hybridized with hGH, HO-1, and GAPDH probes as described previously (21).

Chromatin Immunoprecipitation (ChIP)—HK-2 cells were grown in 15-cm collagen-coated dishes and treated with vehicle (Me₅SO) or hemin (5 μM) for 2 h. Cells were fixed with 1% formaldehyde for 10 min at room temperature, and ChIP was performed as described (21). Normal rabbit serum was used instead of the primary antibody (no antibody) as a negative control. The recovered DNA was amplified for 26 cycles at three different loci. PCR products were electrophoresed, blotted, and hybridized with the corresponding nested oligonucleotides listed in Table 1. Autoradiographs from 3–5 independent assays were scanned using a ScanMaker 9800 XL with Transparency Media adapter (Microtek, Carson, CA) using Adobe Photoshop Elements version 2.0 software (Adobe Systems Inc. San Jose, CA), and densitometry was performed using NIH Image 1.63 software.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared from HK-2 cells stimulated with hemin (5 μM), cadmium (10 μM), or vehicle (Me₅SO) as described previously (48). Binding reactions were electrophoresed in a 0.5% TBE, 5% nondenaturing polyacrylamide gel, fixed, dried, and exposed to film. The G-5 wild-type probe consisted of the following oligonucleotides and its complement annealed, 5'-gctgacagtctgcagcgtgcgtcatgtttggg-3'. The G-5 and G-6 nucleotides are in uppercase left to right, respectively and were mutated to form AT base pairs. Supershifts were performed by adding 2 μg of the indicated antibody after addition of labeled probe and incubated at 22 °C for 15 min. HEK293 cells were grown in 10-cm collagen-coated dishes and transfected with 10 μg of Nrf2-GFP plasmid (kindly provided by Dr. Tony Kong, The State University of New Jersey, Piscataway) for 24 h. Gel shift assays were performed using whole cell extracts described (50) and the mouse StRE oligonucleotide 5'-tttttcgtaggtagatcttaag-3'.
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Western Blot Analysis—For junB<sup>+/+</sup> and junB<sup>−/−</sup> MEFs, nuclear extracts were harvested as described previously (21) for the gel shift assays, and 75 μg of nuclear protein was electrophoresed on a 10% Tris-glycine SDS-polyacrylamide gel. For junD<sup>+/+</sup> and junD<sup>−/−</sup> MEFs, whole cell protein was lysed in RIPA buffer and quantitated using a Bradford assay (Bio-Rad), and 25 μg of total protein was electrophoresed on a 12% Tris-glycine SDS-polyacrylamide gel. After transfer to a polyvinylidene difluoride membrane (Millipore, Billerica, MA), JunB and JunD were detected using 1:200 dilutions of rabbit anti-JunB (N-17, sc-46; Santa Cruz Biotechnology) or rabbit anti-JunD (sc-74; Santa Cruz Biotechnology) antibodies, followed by a horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Protein was visualized using the ECL chemiluminescent detection system (Amersham Biosciences).

Real Time PCR—The junB<sup>+/+</sup>, junB<sup>−/−</sup>, junD<sup>+/+</sup>, and junD<sup>−/−</sup> MEFs were stimulated with vehicle (Me<sub>2</sub>SO) or hemin (5 μM), and RNA was collected at 2 and 5 h as described previously (21). The RNA was then precipitated with 1.75 M lithium chloride on ice for 2 h. An equal volume of 70% ethanol was added, and the RNA was collected by centrifugation. After drying, the RNA was resuspended in diethyl pyrocarbonate-treated water, and its integrity was verified by gel electrophoresis. 3 μg was used in 1st strand cDNA synthesis using a superscript kit (Invitrogen). The cDNA was then diluted to a total of 50 μl, and 1 μl of this was used in amplification. Quantitative real time PCR was performed with 2× SYBR Green Mastermix (Invitrogen) and 900 nm primers for mouse Ho-1 and GAPDH. Cycling was performed on a Applied Biosystems Prism 7900HT sequence detection system with the following cycling parameters: 50 °C for 2 min, Taq activation at 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were performed in triplicate and specificity monitored using melting curve analysis after cycling. Primers used are as follows (5′–3′): GAPDH, 5′-TCCCACTCCTTCACCTCTCA-3′ and 5′-AGTTGGTATGGGCGCT CTCTTG-3′; Ho-1, 5′-AGTTACA CATCCAAGCGGAGA-3′ and 5′-CTCTGGACACCTGACCCTTCT-3′.

Statistical Analysis—Results are expressed as mean ± S.E. For the luciferase activity assay, analyses were performed using analysis of variance and the Student-Newman-Keuls test. All results are considered significant at p < 0.05.

RESULTS
Identification of DNase I-hypersensitive Regions and DMS-protected Guanines in the Human HO-1 Promoter—DNase I hypersensitivity analysis of the HO-1 promoter in human renal proximal tubule cells (HK-2) revealed one constitutive hypersensitive region (HS-1) that maps to −2.2 kb proximal to the indicated HindIII site (Fig. 1A). This region corresponds to an E box (CACGTG) motif at −44/−38 bp we have previously shown to be bound by both USF1 and USF2 in vivo in HK-2 cells (21). No other obvious hypersensitive sites were observed 3′ to the HS-1 site. We also performed DNase I hypersensitivity analysis of distal regions of the HO-1 promoter. As shown in Fig. 1B, three distinct hypersensitive sites denoted as HS-2, HS-3, and HS-4 at approximately −4.0, −7.2, and −9.2 kb of the HO-1 promoter, respectively, were detected. These three HS regions were also constitutive as they were present in both vehicle- and hemin-treated cells. The sequence of the HO-1 promoter in the HS-3 region also contains an E box motif. The HS-4 region is more intense in signal than HS-3 and contains another E box consensus sequence and multiple AP-1 sites. This HS-4 region is required for HO-1 induction by transforming growth factor-β<sup>4</sup> and experiments to determine the factors responsible for the

<sup>4</sup> A. Mark, T. Hock, and N. Hill-Kapturczak, manuscript in preparation.
HS-4-hypersensitive site are currently ongoing. In this study, we have focused on HS-2, because this region is contained within a 4.5-kb promoter construct that has been demonstrated previously to be the minimal promoter responsive to hemin and cadmium (45, 46).

To analyze the HS-2 region at single nucleotide resolution, we performed in vivo DMS footprinting on the transcribed strand of the HO-1 promoter in HK-2 cells. Six guanine bases (open circles referred to as G1–6) were protected from DMS methylation in comparison with their respective signal intensity in the in vitro controls (Fig. 2A). Three guanines (Fig. 2A, closed circles) were hypermethylated. G-1, -2, and -3 reside in a sequence that database searches suggest may be bound by REL factors, including c-Rel, nuclear factor of activated T cells, and NF-κB. G-4 is located within a cAMP response element (CRE) that has been suggested previously to be bound by cAMP-response element-binding protein and contributes to HO-1 promoter activation by oxidized phospholipids (51). The CRE also resembles an AP-1 like sequence and will be referred to as CRE/AP-1. G-5 is within a classic AP-1 sequence (TGACTCA) that extends to form an ARE. G-6 is 2 bp downstream of G-5. DMS footprinting of the nontranscribed strand was also performed and revealed no obvious protections (Fig. 2B). The relative locations and sequences of the G1–6 region are illustrated in Fig. 2C. The consensus StRE sequences corresponding to the mouse HO-1 E1 enhancer region are underlined in Fig. 2C.

Mutational Analysis of the G1–6 DMS Footprints—The six protected guanines were each mutated to an adenine in the pHOGH/4.5 reporter construct containing a 4.5-kb fragment of the human HO-1 promoter. Reporter transcription was assayed in HK-2 cells stimulated with hemin (5 μM) or cadmium (10 μM) by Northern blot hybridization. As shown in Fig. 3A, the only mutation that produced a significant effect on
HO-1-driven promoter activity was G-5. This single mutation decreased HO-1 promoter activity by ~50% in both hemin- and cadmium-treated cells as compared with the wild-type 4.5-kb construct as well as the others shown. The levels of endogenous HO-1 gene expression were assessed to verify hemin- and cadmium-mediated HO-1 induction (Fig. 3A, middle panel). The result for the G-5 mutation was also confirmed using a luciferase reporter system (data not shown).

We observed little or no effect with the G1–4 or the G-6 mutation. An additional control point mutation (G to A) of the guanine nucleotide immediately adjacent to the G-6 footprint (denoted by an asterisk in Fig. 2C) was also tested and revealed no effect on reporter activity (Fig. 3A, lower panel). As stated above, however, because G1–3 and G-4 reside in consensus DNA-binding motifs, we constructed deletions of both these regions. The sequence junctions of the wild-type pHOGH/4.5 and the deletions are shown in Fig. 3B. As shown in Fig. 3C, deletion of the CRE/AP-1 (ΔCRE) resulted in a significant decrease in reporter expression. A densitometric analysis of the Northern data from 3 to 4 independent experiments for the wild-type 4.5-kb HO-1 promoter in comparison with the G-5 mutation and the ΔCRE construct is shown in Fig. 3D. The deletion of G1–3 (ΔG1–3) produced a minimal effect. We also examined double and triple mutant reporter constructs that contained the ΔCRE, E box M3 (the point mutation within the proximal E box that abolished USF binding) (21), and the G-5 single point mutation. As shown in Fig. 3C, the triple mutant (ΔCRE, E box M3, and G-5) completely abolished reporter transcription with hemin and cadmium stimulation. The two double mutants (ΔCRE + G-5 and E box M3 + G-5) tested reduced reporter transcription, but it was still detectable at longer exposures (data not shown). The E box M3 mutation alone partially reduced reporter expression as observed previously (21). Similar results were also obtained with the luciferase reporter (data not shown). These results suggest that HO-1 promoter activity requires the CRE/AP-1 as well as the G-5-promoted guanine in addition to the proximal E box.

Identification of Proteins Binding to the G-5 Guanine—We investigated the importance of the G-5 and G-6 guanine mutations (adenine substituted for guanine) in an EMSA. As shown in Fig. 4A, an obvious protein-DNA complex was observed with the wild-type oligonucleotide probe (2nd lane). The G-6 mutant oligonucleotide was as efficient as the wild-type competitor in gating the importance of the G-5 and G-6 guanine-promoter activity requires the CRE/AP-1 as well as the G-5-promoted guanine in addition to the proximal E box. The G-5 mutant oligonucleotide was as efficient as the wild-type competitor in gating the important of the G-5 and G-6 guanine-promoter activity requires the CRE/AP-1 as well as the G-5-promoted guanine in addition to the proximal E box. The G-5 mutant oligonucleotide was as efficient as the wild-type competitor in gating the important of the G-5 and G-6 guanine-promoter activity requires the CRE/AP-1 as well as the G-5-promoted guanine in addition to the proximal E box. The G-5 mutant oligonucleotide was as efficient as the wild-type competitor in gating the important of the G-5 and G-6 guanine-promoter activity requires the CRE/AP-1 as well as the G-5-promoted guanine in addition to the proximal E box. The G-5 mutant oligonucleotide was as efficient as the wild-type competitor in gating the important of the G-5 and G-6 guanine-promoter activity requires the CRE/AP-1 as well as the G-5-promoted guanine in addition to the proximal E box.
in Fig. 4B, only the pan-Jun antibody produced a reduction in band shift intensity, and a small supershift in nuclear extracts from uninduced (control) or hemin-stimulated HK-2 cells. Both Nrf1 and Nrf2 antibodies failed to show a supershift or signal reduction of the protein-DNA complex. As a positive control for Nrf2 binding, we used whole cell extracts from HEK293 cells overexpressing Nrf2-GFP or mock-transfected cells in gel shift assays. Overexpression of Nrf2 was confirmed by Western blot analysis. Using the mouse StRE oligonucleotide, a faint supershift was observed with the C-20 Nrf2 antibody in cells overexpressing Nrf2 (data not shown).

We also tested other applicable antibodies to transcription factors, including Fra1, Fra2, c-Fos, pan-Maf (MafG, -F, and -K), and the erythroid-specific factor NF-E2 and observed no supershifted complexes (data not shown). We also tested anti-Bach1 polyclonal sera and observed no specific supershift or reduction of the protein-DNA complex (data not shown). We then tested antibodies to the specific Jun proteins c-Jun, JunB, and JunD. Both JunD and c-Jun antibodies clearly supershifted the protein-DNA complex (Fig. 4C). Even though we did not detect any supershift with the JunB antibody, it is still possible that it could interact with the region because JunB has been shown previously to bind DNA with a 10-fold less affinity in vitro than for example c-Jun (52). These in vitro data suggest that Jun proteins, JunD and c-Jun, interact with the ARE/AP-1 sequence within the HO-1 promoter.

**Jun Proteins Associate with the Human HO-1 Promoter in Vivo**—We then determined whether Jun proteins interacted with the HO-1 promoter in vivo. We performed ChIP assays and analyzed regions spanning from +2.2 kb to the HS-2 region which, as indicated in Fig. 1, is located at approximately −4.0 kb from the transcriptional start site of the HO-1 promoter. We used antibodies to c-Jun, JunB, JunD, Nrf2 and USF1, as well as Menin, a tumor suppressor nuclear protein capable of interacting with JunD (53). The results of these analyses are shown in Fig. 5. The +2.2-kb region, where no DNase I hypersensitivity was detected, showed that none of the factors tested associated with this region (Fig. 5A, upper panel). We then examined the proximal promoter region and confirmed that USF1 (positive control) showed a strong association; however, we also detected a significant signal with JunD in the vehicle-treated lane but not following hemin induction (Fig. 5A, 2nd panel). In the −4.0-kb region, we observed a significant association with JunB (in vehicle and hemin-treated samples) and USF1 and to a lesser extent with JunD and Nrf2 (in vehicle-treated samples) (Fig. 5A, lower panel). A quantitative analysis of the ChIP results for the −4.0-kb region from 3 to 5 independent experiments is shown in Fig. 5B.

**FIGURE 4.** Jun proteins bind to the ARE in vitro in the HS-2 site. A, nuclear extracts were prepared from vehicle (Me2SO)-treated HK-2 cells and incubated with a wild-type (WT) probe containing the G-5/AP-1 site from the human HO-1 promoter. The arrow indicates the shifted DNA-protein complex. A 30-fold excess of cold competitor (CC) was added as indicated consisting of either the cold probe itself (WT) or the G-5 and G-6 mutant probes. NS = nonspecific binding. B, EMSA performed using the same probe as in A using the indicated antibodies for supershift experiments. Nuclear extracts were derived from HK-2 cells treated with 10 μM cadmium, 5 μM hemin, or control (Me2SO) for 2 h. C, EMSA using nuclear extracts from 5 μM hemin or control (Me2SO)-treated HK-2 cells. The wild-type probe was used in these experiments. Specific Jun antibodies (Ab) used are indicated, and supershifts are marked with an asterisk. Cold competitors (CC) used are in 50-fold excess of labeled probe.
Overexpression of Jun Proteins and HO-1 Reporter Expression—Co-transfection studies were performed using c-Jun, JunB, and JunD expression plasmids and the 4.5-kb HO-1 promoter construct. Both c-Jun and JunB increased promoter activation, whereas JunD inhibited this induction by about 50% (Fig. 6, A and B).

Effects of Overexpression of Jun Proteins on Mutated HO-1 Reporter Constructs—To establish whether the effects of JunB were mediated through one or more of the identified regulatory elements, co-transfection studies were performed using a JunB expression plasmid and mutations of the 4.5-kb HO-1 promoter construct in HK-2 cells. As shown in Fig. 7, overexpression of JunB in HK-2 cells resulted in a significant increase in reporter activity with the wild-type pHOGL3/4.5 construct in vehicle and hemin-treated cells. This increase was not affected in the ΔCRE mutant reporter. However, the double mutant consisting of the ΔCRE with the G-5 point mutation caused a significant reduction in JunB-inducible reporter activity, suggesting that JunB may be acting through the G-5 guanine in HK-2 cells. Using the triple mutation (ΔCRE+G-5+E boxM3), JunB-inducible reporter activity was also significantly reduced. Similarly, we tested the effects of JunB and JunD overexpression in T47D cells. As shown in Fig. 7, JunB augmented and JunD reduced reporter expression of the wild-type 4.5-kb construct following hemin stimulation. These effects were significantly reduced in the presence of the ΔCRE mutasion, suggesting that in T47D cells, both JunB and JunD may be acting through the CRE/AP-1 site. These results were also observed with the double and triple mutant constructs.

Effect of JunB and JunD on Endogenous HO-1 Expression in HK-2 Cells—We focused our attention on JunB and JunD because both showed an association with the HO-1 promoter and modulated HO-1 promoter activity. As shown in Fig. 6A (middle panel), we did not observe any obvious differences in HO-1 mRNA induction with Jun overexpression, and we attribute this to the relatively low transfection efficiency of HK-2 cells. To address the effects of JunB and JunD overexpression on endogenous HO-1 expression in HK-2 cells, immunocytochemistry was performed. A significant number of JunB-overexpressing cells (77.6 ± 2.1%) were positive for HO-1 expres-
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Figs. 6 and 7. Taken together, these results demonstrate that results are consistent with the hemin stimulation (Fig. 8, cells (79.8 ± 80%) was negative for HO-1 expression following hemin stimulation (Fig. 8, A, right lower panel, and C). These results are consistent with the HO-1 promoter assays shown in Figs. 6 and 7. Taken together, these results demonstrate that JunB and JunD have opposing effects; JunD is a repressor whereas JunB enhances HO-1 promoter activity and endogenous HO-1 expression in human renal epithelial cells.

Hemin-induced HO-1 Expression in JunB- and JunD-deficient Fibroblasts—To confirm the involvement of Jun proteins in HO-1 regulation, HO-1 mRNA levels were evaluated by real time PCR using junB+/− and junB−/− as well as junD+/− and junD−/− MEFs treated with vehicle (Me2SO) or hemin. As shown in Fig. 9A, junB+/− cells showed an 83 and 59% decrease in HO-1 mRNA levels at 2 and 5 h, respectively, following hemin stimulation, as compared with junB−/− cells (p < 0.001). Conversely, junD−/− cells showed a 15% increase in HO-1 mRNA levels at 2 h, which was significantly higher (64%) at 5 h (p < 0.05), in comparison with junB+/− cells (Fig. 9B). These results provide conclusive evidence for the positive and negative regulatory roles for Jun proteins in the molecular regulation of HO-1 induction.

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DISCUSSION

In this work we present a comprehensive evaluation of the regulatory regions of the human HO-1 gene using DNase I hypersensitivity and in vivo DMS footprinting in human renal epithelial cells. Coupled with our previous in vivo analysis of the proximal E box sequence (21), this work provides new insights into the molecular regulation of human HO-1 gene expression. We have identified four DNase I HS regions in the human HO-1 promoter. HS-3 and HS-4 are located distal to the HS-2 regulatory region, and each contains a single consensus E box motif. Thus, there are a total of three E box sequences in the HO-1 promoter, and each of them is associated with a constitutive DNase I HS site. The DMS footprinting of HS-2 illustrates that this region is rich in DNA-binding proteins as determined by the presence of six DMS protected guanines in three different consensus sequences. The first region in HS-2, G1–3, is bound by an unknown factor(s) and when deleted results in only a modest decrease of hemin or cadmium-mediated induction (Fig. 3C). The second region, G-4, is contained within a CAMP-response/ AP-1 element (CRE/AP-1) and contributes significantly to HO-1 promoter activation (Fig. 3C). The third region (HS-2), located within a classical AP-1 site, is bound by Jun proteins in vitro (Fig. 4, B and C) and in vivo as suggested by our ChIP results (Fig. 5). Two of these regulatory regions, G-4 and G-5, as well as the E box at −44 bp synergize for maximal hemin and cadmium-induced HO-1 promoter activity (Fig. 3C). We also show that JunB overexpression is capable of activating the HO-1 promoter, whereas JunD negatively regulates promoter activity (Figs. 6 and 7). Furthermore, overexpression of JunB increases endogenous HO-1 protein, and JunD inhibits hemin-induced HO-1 expression in HK-2 cells (Fig. 8). The opposing effects of JunB and JunD were also observed in mouse embryonic fibroblasts genetically deficient for these proteins (Fig. 9) underscoring their important role in providing the cell with a means of modulating HO-1 expression.

Our results demonstrate that JunB associates with the HS-2 region and JunD with the proximal promoter region (HS-1) preferentially in the uninduced state. JunD also associates with the HS-2 region, although this interaction is lower than that for JunB. The results of our co-transfection studies (Fig. 7) using mutant reporters are also suggestive that JunB and JunD exert their effects at the CRE/AP-1 and the G-5 guanine. However, as shown in Fig. 9A, HO-1 expression, albeit significantly reduced, was still inducible in JunB-deficient cells, suggesting that other co-factors may be involved. These could include c-Jun, USF, Nrf2, Menin, and possibly others. The observation of JunD interacting with the proximal promoter is intriguing because there are no canonical AP-1 sites in this region, the nearest being −4 kb upstream from the HS-1 site in HS-2. It is possible that long range chromatin interactions and “capturing” of JunD in the formaldehyde cross-linking step of the ChIP assay are responsible for this finding. The appearance of USF1 at HS-2 in the ChIP assay, even though there is no E box sequence in this region, would suggest an interaction between the HS-1 and HS-2 regions. This seems plausible given that we detect far distal hypersensitive sites in the HO-1 promoter. To confirm this phenomenon, experiments to Capture Chromosome Conformation (3C assay) (54) are currently being pursued in our laboratory.

The cap ‘n’ collar transcription factor, Nrf2, has been proposed to be the principal regulator that mediates mouse Ho-1 gene activation through interaction with the ARE sequence(s) (7). Overexpression of Nrf2 is capable of activating the 4.5-kb human HO-1 promoter in HK-2 cells (data not shown). However, our results with induction of the human HO-1 gene cannot be explained entirely by the involvement of Nrf2 for several reasons. First, we were unable to show Nrf2 binding to the G-5 probe on gel shift-supershift assays. These findings are consistent with the observations of Kronke et al. (51), who were unable to supershift the human ARE sequence with Nrf2 antibodies. Second, Nrf2 does not conclusively associate with the HS-2 region containing the ARE sequence. Third, mutations in multiple regulatory sequences, including the ARE, CRE/AP-1, and E box, were required to abolish HO-1 promoter responsiveness. It is possible that Nrf2 activates the human HO-1 promoter indirectly through AP-1 family members as shown recently for the rat glutamate-cysteine ligase catalytic subunit gene (55). A comparison of the HS-2 region with the previously described E1 enhancer region located at −4.0 kb of the mouse Ho-1 gene (7, 56) reveals that the three StRE sequences of the mouse E1 region (Fig. 2C, underlined) closely align with the analogous region in the human HO-1 promoter. The G-4 and G-5 footprints are within the corresponding mouse HO-1 StRE sequences. However, to the best of our knowledge, in vivo footprints of these regions in the mouse Ho-1 gene has not been reported.

Our findings demonstrate that the detected HS sites and the in vivo footprints in the human HO-1 promoter are constitutive. There are also no hypersensitive sites or DMS-protected guanines that appear upon addition of hemin. However, mutational analysis of at least some of the footprints alone or in combination resulted in a functionally significant effect on stimulus-dependent HO-1 promoter activity. The question then arises as to how HO-1 induction is triggered given that these regulatory regions are constitutively bound.

FIGURE 9. HO-1 expression in JunB- and JunD-deficient MEFs. SYBR Green incorporation real time PCR was performed on samples derived from junB+/+ and junB−/− (A) as well as junD+/+ and junD−/− (B) MEFs that were induced with vehicle (MeSO3) or 5 µM hemin for the indicated time points. The reactions were performed in triplicate. Results shown are fold increase over vehicle-treated cells and were normalized to the fold increase seen in wild-type JunD MEFs. Insets, the absence of JunB and JunD was verified by Western blotting using nuclear extracts for Junb and whole cell lysates for JunD cells, as described under “Experimental Procedures.” * p < 0.001 versus junB+/+; **, p < 0.05 versus junD+/+.
Several mechanisms may account for these observations. The ChIP findings showing the presence of JunD in the uninduced state and its absence following hemin stimulation (Fig. 5) provide one level of control. Whether JunD interacts with USFs or the basal transcriptional machinery remains to be elucidated. It is also reasonable to consider the effects of hemin stimulation on upstream signaling cascades that alter phosphorylation of proteins or protein–protein interactions. In addition, histone modifications could also contribute to the epigenetic control of HO-1 gene expression. Furthermore, chromatin looping may bring long range enhancers close to critical regulatory regions in the promoter following stimulation as has been shown for the β-globin locus (57).

We have described previously an internal enhancer that in conjunction with the −4.5-kb HO-1 promoter activates hemin- and cadmium-stimulated reporter gene expression to levels significantly higher than the −4.5-kb promoter alone in human endothelial and renal epithelial cells (46). However, DNase I-hypersensitive studies have been unable to detect any hypersensitive sites in the introns of the HO-1 gene. It is possible that the chromatin in the intronic region is open, and hence all regions are always equally accessible to DNase I, making it difficult to be identified by this assay. By sequential 5′ and 3′ deletions of this previously described large 12.5-kb internal enhancer, we have delineated a 220-bp intronic region of the human HO-1 gene that functions as the minimal enhancer and more importantly requires critical sequences in the HS-2 region of the human HO-1 promoter described in this work. Further studies to characterize the interaction between this minimal intronic sequence and the 4.5-kb HO-1 promoter and its role in human HO-1 gene regulation are in progress. The results of our studies are not applicable to all HO-1 inducers. For several other stimuli, including oxidized lipids and trans-

**Acknowledgments**—We are grateful to Jean-Michel Mesnard for the JunD expression vectors, Rik Derynck for the c-Jun expression plasmids, Moshe Yaniv and Sunita Agarwal for the JunD-deficient mouse embryonic fibroblasts, and Tony Kong for the Nrf2- GFP expression plasmid. We are grateful to Tim Townes for help with the real time PCR studies. We thank Harry Nick, Paul Sanders, Nathalie Hill-Kapturczak, and Jessy Deshane for helpful suggestions.

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