Sp1 Regulates Chromatin Looping between an Intrinsic Enhancer and Distal Promoter of the Human Heme Oxygenase-1 Gene in Renal Cells* [S]

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HO-1 (heme oxygenase-1) is an inducible microsomal enzyme that catalyzes the degradation of pro-oxidant heme. The goal of this study was to characterize a minimal enhancer region within the human HO-1 gene and delineate its role in modulating HO-1 expression by participation with its promoter elements in renal epithelial cells. Deletion analysis and site-directed mutagenesis identified a 220-bp minimal enhancer in intron 1 of the HO-1 gene, which regulates hemin-mediated HO-1 gene expression. Small interfering RNA, decoy oligonucleotides, site-directed mutagenesis, and chromatin immunoprecipitation assays confirmed the functional interaction of Sp1 with a consensus binding sequence within the 220-bp region. Mutations of regulatory elements within the −4.5 kb promoter region (a cyclic AMP response and a downstream NF-E2/AP-1 element, both located at −4.0 kb, and/or an E-box sequence located at −44 bp) resulted in the loss of enhancer activity. A chromosome conformation capture assay performed in human renal epithelial cells (HK-2) demonstrated hemin-inducible chromatin looping between the intrinsic enhancer and the −4.0 kb promoter region in a time-dependent manner. Restriction digestion with ApaI (which cleaves the 220-bp enhancer) led to a loss of stimulus-dependent chromatin looping. Sp1 small interfering RNA and mithramycin A, a Sp1 binding site inhibitor, resulted in loss of the loop formation between the intrinsic enhancer and the distal HO-1 promoter by the chromosome conformation capture assay. These results provide novel insight into the complex molecular interactions that underlie human HO-1 regulation in renal epithelial cells.

HO-1 (heme oxygenase-1) is an antioxidant defense enzyme that breaks down free heme to bilirubin, carbon monoxide and iron (1). HO-1 is highly inducible in most cell types and exhibits potent anti-inflammatory, antifibrotic, vasodilatory, and antiapoptotic properties (2, 3). In addition to supporting a vital function in maintaining tissue homeostasis, the induction of HO-1 by chemical inducers confers cytoprotection against oxidative tissue injury (2, 4). This protective effect is also elicited by selective overexpression of HO-1 in many in vitro and in vivo model systems and also corroborated by studies in HO-1 knock-out mice (5–12).

The molecular regulation of HO-1 induction by most stimuli, including heme, is controlled at the transcriptional level (reviewed in Ref. 13). HO-1 has been studied as a model for redox regulation of transcriptional activation. In addition to serving as a substrate for the HO-1 enzyme, heme is also a potent inducer of the HO-1 gene in vitro (14–16) and in vivo (8). Both positive and negative control elements within the HO-1 promoter have been described (17). Multiple signaling molecules and transcriptional regulators have been shown to participate in the regulation of the mouse HO-1 gene (reviewed in Refs. 13 and 17).

Previous studies described a 12.5-kb enhancer internal to the HO-1 gene (15). This region, in conjunction with the −4.5 kb HO-1 promoter conferred significant hemin-mediated induction of reporter activity in human aortic endothelial cells as well as human renal proximal tubule epithelial cells (15). Enhancer activity was orientation-independent, was unable to function with a heterologous thymidine kinase promoter, and required a region between the −3.5 and −4.5 kb region of the human HO-1 promoter. Interestingly, the enhancer region was not functional for all inducers of HO-1 and failed to respond to transforming growth factor-β, hydrogen peroxide (H₂O₂), or oxidized lipids (15), indicating a partial specificity of the enhancer for certain stimuli that could potentially involve a common mechanism with similar regulatory elements.

The present study investigated regulatory sequences and molecular mechanisms involving the internal enhancer. Using sequential 5′ and 3′ deletions of the 12.5-kb enhancer fragment, we delineated a 220-bp region that was in intron 1 and extended into exon 2. In addition, we demonstrate the participation of the transacting regulatory protein, Sp1 (specificity protein 1), with this minimal enhancer in response to hemin stimulation. Mutational analysis of key regulatory elements (cyclic AMP-response element (CRE), 3

The abbreviations used are: CRE, cAMP-response element; 3C, chromosome conformation capture; ChIP, chromatin immunoprecipitation; HK-2, human renal proximal tubular cells; HS, hypersensitive site; NF-xB, nuclear factor-xB; siRNA, small interfering RNA; WT, wild type; BAC, bacterial artificial chromosome.

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NF-E2/AP-1, and E-box) in the human HO-1 promoter indicated that enhancer activity requires potential interaction of these promoter sequences with the intronic enhancer. Furthermore, stimulus-specific chromatin loop formation between the intronic enhancer and the HO-1 promoter region was confirmed by the chromosome conformation capture (3C) assay and found to be dependent on the transcription factor, Sp1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HK-2 cells (ATCC, Manassas, VA), an immortalized human renal proximal tubule epithelial cell line, were maintained in keratinocyte serum-free medium supplemented with 5 ng/ml recombinant epidermal growth factor and 40 μg/ml bovine pituitary extract and 1% antibiotic-antimycotic solution (Invitrogen) in collagen-coated tissue culture dishes as described earlier (18). HEK293 (Human embryonic kidney 293) cells (ATCC) were passaged in Dulbecco’s modified Eagle’s medium/F-12 medium (Mediatech) with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, nonessential amino acids (100 μM), gentamycin (30 μg/ml), and amphotericin B (15 ng/ml). All cells were grown at 37°C in 95% air and 5% CO2.

**Plasmid Constructs**—The construction of the −4.5 kb human HO-1 promoter fragment (pHOGL3/4.5) and the +12.5 kb enhancer with the −4.5 kb HO-1 promoter have been previously described (15). Briefly, the −4.5 kb 5′-flanking region of the human HO-1 gene was generated by long range PCR and cloned into the BglII site of a luciferase reporter (pGL3), resulting in the pHOGL3/4.5 construct. The entire +1 to +12.5 kb region of the human HO-1 gene (including all exons and introns) was generated using long range PCR and cloned into the Sall site of pHOGL3/4.5, resulting in the pHOGL3/4.5 +12.5 construct (supplemental Fig. 1).

The pHOGL3/4.5 +12.5 construct was used as the parental clone for the generation of 5′ and 3′ deletions of the +12.5 kb enhancer. Briefly, the pHOGL3/4.5 +12.5 construct was first digested with Sall to release the 12.5-kb insert. The 12.5-kb insert was then incubated with Bal3I exonuclease for increasing amounts of time to generate 5′ and 3′ deletion fragments. These enhancer deletion fragments were then cloned into the Sall site of pHOGL3/4.5, upstream of the −4.5 kb promoter (supplemental Fig. 1 and Fig. 1A). The 3′ deletion of a 4.0-kb fragment (Δ4.0) from the +12.5 kb enhancer resulted in a +8.5 kb enhancer fragment that was cloned into pHOGL3/4.5 to generate pHOGL3/3′Δ4.0. A 5′ deletion of a 2.0-kb region (Δ2.0) resulted in a +10.5 kb enhancer fragment that was cloned into pHOGL3/4.5 to generate pHOGL3/3′Δ+2.0. Likewise, a 5′ deletion of a 1.1-kb fragment (Δ1.1) resulted in a +11.4 kb enhancer fragment that was cloned into pHOGL3/4.5 to generate pHOGL3/5′Δ1.1 (supplemental Fig. 1 and Fig. 1A). The construct pHOGL3/3.5 +12.5 represents the +12.5 kb internal enhancer containing the −3.5 kb region of the human HO-1 promoter and has been described previously (15).

An 866-bp region of the enhancer, located between the Δ2.0 and Δ1.1 region at the 5′-end of the +12.5 kb enhancer fragment (in intron 1 with 60 bp at the 3′-end overlapping with exon 2 of the human HO-1 gene) was amplified by PCR and cloned into TA vector. The purified product was then cloned in both orientations into the Sall site of pHOGL3/4.5 to generate pHOGL3/4.5 +866 (Fig. 1C). A 220-bp fragment at the 3′-end of the 866-bp region containing a putative Sp1 binding site was amplified by PCR and cloned into the Sall site of pHOGL3/4.5 to generate pHOGL3/4.5 +220 (Fig. 1C). All of the enhancer fragments, including the 866- and 220-bp fragments, were cloned into the Sall site of pHOGL3/4.5 upstream of the −4.5 kb promoter (supplemental Fig. 1). All constructs were verified by sequencing.

**Transient Transfection with Plasmid Constructs**—HK-2 cells in 10-cm dishes were transiently transfected using Lipofectamine 2000 (Invitrogen) with equimolar amounts of plasmid DNA using a batch transfection method as described earlier (18). Transfections were performed for 2 h, cells were rinsed once with Hanks’ balanced salt solution, and fresh growth medium was added. After a 16-h recovery, cells from each 10-cm dish were split onto two 12-well collagen-coated dishes. Cells were treated with vehicle (DMSO) or hemin (5 μM) for 16 h, and cell lysates were collected for luciferase activity measurements using a luminometer.

**Stimulation of Cells with Hemin and Mithramycin A**—Hemin (heme chloride) was used at a final concentration of 5 μM from a 1 mM stock solution in DMSO. An equal volume of vehicle, DMSO, was used as a control for all of the induction experiments. Cells were treated with mithramycin A (Sigma) (10 and 100 nM), a GC binding site inhibitor, for 16 h prior to hemin treatment. An equal volume of vehicle (methanol) was used as a control for the pretreatment.

**Site-directed Mutagenesis**—The plasmid pHOGL3/4.5 +220 was used as the parental clone for all of the mutagenesis experiments. Mutations in the Sp1 and CEBP-α sites in the +220 bp enhancer (Fig. 2A) were generated using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s recommendations. The plasmid constructs with mutations in the −4.0 kb promoter region (deletion of the CRE binding site (ΔCRE), a single nucleotide mutation in the NF-E2/AP-1 element termed ARE-5 (antioxidant response element 5)), and mutations in the −44 bp promoter region (a single nucleotide mutation in the E-box element labeled M3) have been described previously (18, 19) (Fig. 3A). All of the mutations and deletions were verified by sequence analysis.

**Sp1 and Sp3 siRNA Studies**—For Sp1 and Sp3 RNA interference analysis, an siRNA pool consisting of four different sequences designed to target Sp1 and Sp3, respectively (siGENOME SMART pool, 400 nm, Dharmacon RNAi Inc., Waltham, MA) or a nontargeting oligonucleotide as a mock siRNA (AAUAGAAAGACACCUCCACUC, 400 nm) control was used. HK-2 and HEK293 cells were transfected with these siRNAs at the indicated concentrations using Oligofectamine reagent (Invitrogen). Oligofectamine alone was used as an additional control for transfections. Following transfections, cells were allowed to recover for 24 h before transfection with the
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pHOGL3/4.5+220 plasmid construct followed by treatment with hemin (5 µM) for 16 h, and luciferase activity was measured as described previously. Immunoblot analysis was performed using anti-HO-1 (SPA-896, Stressgen), anti-Sp1 (SC-420), anti-Sp3 (SC-644) (Santa Cruz Biotechnology), and anti-actin (Sigma) antibodies as described previously (18). For the 3C assay, cells were allowed to recover for 48 h in normal growth medium and then incubated in culture medium containing 1% fetal bovine serum for 16 h prior to hemin treatment.

Decoy Oligonucleotide Transfection Assays—The sequences of the forward strand for the wild-type (WT) and Sp1-mutated (Mut decoy) decoy oligonucleotides are described in Fig. 2D. Double-stranded oligonucleotides were prepared by adding equal amounts of forward and reverse strand oligonucleotides in annealing buffer (10 mM Tris (pH 8), 1 mM EDTA, and 50 mM NaCl), boiling for 10 s, and then cooling slowly overnight at room temperature. The double-stranded oligonucleotides were then cotransfected in HK-2 cells together with the pHOGL3/4.5+220, using a 200-fold excess of the double-stranded decoy oligonucleotides versus the reporter construct. The cells were then treated with DMSO or hemin (5 µM) and assayed for luciferase activity.

Chromatin Immunoprecipitation (ChIP)—For the ChIP assay, the ChIP-IT Express kit (Active Motif, Carlsbad, CA) was used as indicated in the manufacturer’s protocol. Briefly, HK-2 cells were grown to 80% confluence in six collagen-coated 15-cm dishes. Hemin (5 µM) and vehicle (DMSO) were added to three dishes each for 2 h. Cells were fixed with 1% formaldehyde for 10 min at room temperature. Plates were washed with ice-cold phosphate-buffered saline, and the fixation was stopped by adding glycine stop-fix solution for 5 min at room temperature. Each plate was washed with ice-cold phosphate-buffered saline then cells were harvested. The pooled cells were pelleted by centrifugation at 10,000 × g for 10 min at 4 °C. Cells were lysed in lysis buffer for 30 min on ice. Then nuclei were released by using a Dounce homogenizer, and extracted nuclei were pelleted by centrifugation. Nuclear extract was digested with enzymatic shearing mixture for 12 min at 37 °C. The enzyme reaction was stopped by adding EDTA, and then the sheared chromatin was collected after centrifugation for 10 min at 4 °C. 2 µg of Sp1 (SC-420, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)) and Sp3 (SC-644, Santa Cruz Biotechnology, Inc.) antibody as well as IgG controls were used for pull-down in 7 µg of sheared chromatin. After a 16-h incubation at 4 °C, magnetic beads were washed once with ChIP buffer 1 and twice with ChIP buffer 2. Cross-linking was reversed, and chromatin was eluted by adding reverse cross-link buffer. Real-time PCR (Applied Biosystems) was performed at least three times with triplicates at each time to amplify the recovered DNA at three different loci. 40 cycles of real-time PCR with a SYBR GreenER quantitative PCR kit (Invitrogen) with appropriate primers (Table 1) was performed. Quantity was calculated by using a signal/noise ratio of ∆∆Ct of input to samples and normalized to IgG controls.

Chromosome Conformation Capture (3C) Assay—The 3C assay was performed according to the method described earlier (20, 21). Briefly, HK-2 cells grown to 90% confluence in 15-cm collagen-coated dishes were treated with hemin (5 µM) or vehicle (DMSO) for 2 h. Additional time points following hemin stimulation were also examined from 0 to 16 h. 3C assays were also performed on HEK293 cells for the Sp1 siRNA knockdown experiments. Cells were washed with cold phosphate-buffered saline twice, and 2% formaldehyde was added to the cells for 10 min at room temperature for cross-linking. The cross-linking was terminated by using one-twentieth volume of 2.5 M glycine. Cross-linked cells were washed with ice-cold phosphate-buffered saline and lysed for 1 h with ice-cold lysis buffer containing 10 mM Tris (pH 8.0), 10 mM NaCl, 0.2% Nonidet P-40, and 1 mM dithiothreitol. The nuclei of the cells were then harvested and suspended in the appropriate restriction enzyme buffer containing 0.3% SDS and incubated at 37 °C for 1 h with gentle shaking. SDS was then sequestered from the samples by the addition of 1.8% Triton X-100 (Fisher), and the samples were incubated at 37 °C for 1 h. The samples were digested with BglII and/or ApaLI restriction enzyme (New England Biolabs) at 37 °C for 16 h. Restriction enzymes were inactivated by the addition of 1.6% SDS and further incubation at 65 °C for 20 min. Samples were diluted with T4 DNA ligase buffer (New England Biolabs) to achieve ∼3 ng of DNA/µl, and then 200 units of T4 DNA ligase (New England Biolabs) were added and incubated for 4 h at 16 °C. Then samples were incubated with Triton X-100, followed by incubation with Proteinase K (200 µg/ml) at 65 °C for 16 h to reverse the cross-linking. This was followed by the addition of 10 µg of RNase/ml, and the DNA was purified by phenol/chloroform extraction. 300 ng of DNA was then analyzed by 35 cycles of two rounds of nested PCR (Eppendorf) with Hotstart Taq polymerase (Qiagen). The PCR products were resolved on 1.5% agarose gel, and the positive bands were cut out. DNA was purified from the gel by using the QIAquick gel extraction kit (Qiagen) and then cloned in TOPO-TA vector (Invitrogen) following the manufacturer’s protocol and sequenced to confirm the ligation of the two distal fragments. To quantify the cross-linking efficiency between fragments, quantitative real-time PCR was performed with SYBR GreenER quantitative PCR Mastermix (Invitrogen) and corresponding primers listed in Table 1. Cycling was performed on an 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, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were performed in triplicate, and specificity was monitored using melting curve analysis after cycling. Cross-linking efficiency was calculated by normalizing the relative Ct values (∆∆Ct) from samples to that from the internal control region, which does not have any restriction enzyme cleavage site used in this assay. A human bacterial artificial chromosome (BAC) containing the HO-1 gene (GenBank™ accession number Z82244) was used as a positive control for the 3C assay. All of the primers used did not contain any repetitive sequences.

Real-time PCR—Total RNA was isolated from cells by TRIzol (Invitrogen), and SYBR Green-based real-time PCR was performed on cDNA first strand synthesis products generated from total RNA (Invitrogen). The reactions were performed in triplicate. Real-time primers are described in Table 1.

Statistical Analysis—All of the experiments were performed at least three times. Results are expressed as mean ± S.E. and
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RESULTS

Deletion Analysis of the Internal Enhancer Reveals an Intronic Region That Functions as the Minimal Enhancer—To delineate the minimal enhancer region, initial studies generated three deletions of the +12.5 kb enhancer luciferase construct (labeled pHOGL3/4.5 + 12.5), including 1.1- and 2.0-kb deletions from the 5′-end and a 4.0 kb deletion from the 3′-end (Fig. 1A). These constructs were transfected into HK-2 cells using equimolar amounts of DNA to control for effective lengths of the plasmids. Transfection of the pHOGL3/4.5 + 12.5 enhancer construct into HK-2 cells demonstrated a ~5-fold increase in reporter activity compared with ~2-fold seen with the pHOGL3/4.5 (promoter only) construct following hemin stimulation, a known inducer of HO-1 (Fig. 1B). Deletion of a 4-kb fragment from the 3′-end of the +12.5 kb enhancer did not significantly affect hemin-mediated activation of reporter activity. Deletion of a 2.0-kb but not a 1.1-kb fragment from the 5′-end of the +12.5 kb enhancer resulted in loss of enhancer activity, suggesting that a region (866 bp) between the 5′- and 3′-ends of the +12.5 kb enhancer in HK-2 cells are derived from three independent experiments. Student’s t test and analysis of variance with Student-Newman-Keuls post-test was used for comparisons. All results are considered significant at $p < 0.05$.

Deletion of a 2.0-kb but not a 1.1-kb fragment from the 5′-end of the +12.5 kb enhancer resulted in loss of enhancer activity, suggesting that a region (866 bp) between the 5′- and 3′-ends of the +12.5 kb enhancer in HK-2 cells were transfected in a 10-cm dish with the 5′-end and a 4.0 kb deletion from the 3′-end (Fig. 1A). These constructs were transfected into HK-2 cells using equimolar amounts of DNA to control for effective lengths of the plasmids. Transfection of the pHOGL3/4.5 + 12.5 enhancer construct into HK-2 cells demonstrated a ~5-fold increase in reporter activity compared with ~2-fold seen with the pHOGL3/4.5 (promoter only) construct following hemin stimulation, a known inducer of HO-1 (Fig. 1B). In other experiments, transfection of HK-2 cells with a pHOGL3/3.5 + 12.5 construct that contained only the −3.5 kb of the HO-1 promoter and the entire +12.5 kb enhancer resulted in a complete loss of hemin-stimulated reporter activity, suggesting that the region between −3.5 and −4.5 kb of the promoter is essential for enhancer function; these findings were consistent with observations in primary human renal proximal tubular cells (15).

We then focused our efforts to characterize the 866-bp region between +1.1 and +2.0 kb. This region is mainly part of intron 1 of the human HO-1 gene with the 3′-end of this enhancer region (60 bp) overlapping with the exon 2 of the human HO-1 gene (Fig. 1C). Hemin induced reporter activity of the 866-bp construct with the −4.5 kb promoter (pHOGL3/4.5 + 866) (Fig. 1D), although this was significantly less than the entire +12.5 kb enhancer (Fig. 1D), similar to the +1.1 kb deletion construct, pHOGL3/3′Δ1.1 (Fig. 1B), indicating that additional sequences in the 3′-end of the 12.5-kb region may also be required for enhancer activity. Similar to the +12.5 kb human HO-1 promoter with the intact +12.5 kb enhancer or with the indicated deletions (shown in open rectangles). The five exons are indicated (E1–E5). The restriction sites for BamHI (B), PstI (P), XbaI (X), and EcoRI (R) are indicated in the genomic map. The enhancer deletion constructs were derived from the parental plasmid, pHOGL3/4.5 + 12.5. Note that all enhancer fragments are cloned upstream of the −4.5 kb promoter in the SalI site of pGL3 as described under “Experimental Procedures.” HK-2 cells were transfected with the indicated constructs in a 10-cm dish using equimolar amounts of DNA and a batch transfection protocol as described under “Experimental Procedures.” 24 h after transfection, cells were split into two 12-well dishes and exposed to control (DMSO) or hemin (5 μM). Luciferase activity was measured 16 h later. Data are represented as mean ± S.E. (error bars) from four independent experiments with 8 replicates/group. *, $p < 0.01$ versus pHOGL3/4.5 + 12.5 hemin-stimulated samples. C, schematic showing the location of the 866- and 220-bp enhancer fragments for the pHOGL3/4.5 + 866 and pHOGL3/4.5 + 220 constructs, respectively. D, 866- and 220-bp intronic enhancer fragments show comparable reporter activity. Cells were transfected and treated with control (DMSO) or hemin (5 μM) as in B. Luciferase activity was measured 16 h later. Values are mean ± S.E. (error bars), n = 3 independent experiments with 8 replicates/group. *, $p < 0.01$ versus pHOGL3/4.5 + 12.5 hemin-stimulated samples; #, $p < 0.05$ versus pHOGL3/4.5 + 12.5 hemin-treated samples. E, orientation-independent effects of the intronic enhancer. Cells were transfected in a 10-cm dish with the 5′−3′ and 3′−5′ orientations of the 866-bp enhancer. Cells were exposed to control (DMSO) or hemin (5 μM), and luciferase activity was measured 16 h later. Values are mean ± S.E., n = 2 independent experiments with 8 replicates/group. *, $p < 0.001$ versus control, vehicle-treated samples.
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220-bp region encompassing 160 bp of intron 1 and 60 bp of exon 2 that was cloned into the Sall site of pHOGL3/4.5 to generate pHOGL3/4.5 +220 (Fig. 1C). Transfection studies with this shorter 220-bp region with the −4.5 kb promoter using pHOGL3/4.5 +220 recapitulated levels of reporter activity following induction with hemin comparable with that observed earlier with pHOGL3/4.5 +866 (Fig. 1D).

Trans-acting Proteins Interact with the Enhancer Region to Promote HO-1 Expression in Response to Hemin—Software-assisted analysis (TRANSFAC, gene regulation data base) of the 220-bp enhancer region for putative transcription factor binding sites revealed the presence of Sp1 and CEBP-α binding sites as depicted in Fig. 2A. Although different from the classical Sp1 consensus motif (CCCGGCC), the Sp1 sequence “CCACCCC” found in the 220-bp HO-1 enhancer has been previously reported to bind Sp1 and other members of the Krüppel-like factor family of transcription factors in other genes (22–25). Site-directed mutagenesis of the Sp1 site followed by transfection of HK-2 cells with the pHOGL3/4.5 +220 construct containing the Sp1 mutation resulted in decreased luciferase reporter activity compared with the wild-type pHOGL3/4.5 +220 construct (Fig. 2B). In addition, knockdown of Sp1 using siRNA inhibited hemin-induced luciferase activity by the pHOGL3/4.5 +220 construct (Fig. 2C).

Further validation of Sp1 was carried out by testing the effect of decoy oligonucleotides on enhancer-mediated activation of the HO-1 reporter. Co-transfection of a 200-fold excess of double-stranded WT decoy oligonucleotides, which contained the putative Sp1 consensus binding sequence, with pHOGL3/4.5 +220 resulted in significant inhibition of hemin-mediated activation of luciferase activity, whereas mutated (Mut decoy) decoy oligonucleotides had no effect (Fig. 2D). These results suggested that the Sp1 binding sequence was important for hemin regulation of enhancer activity.

FIGURE 2. RNA interference and site-directed mutagenesis of Sp1 binding sites attenuate enhancer mediated reporter activity in HK-2 cells. A, schematic of the target sequences of Sp1 and CEBP-α for site-directed mutagenesis is shown for the wild-type as well as the mutated pHOGL3/4.5 +220 constructs containing mutations in the Sp1 and CEBP-α sequences. Data shown represent luciferase activity determined following stimulation with control (DMSO) or hemin (5 μM). Values are means ± S.E. (error bars), n = 3 independent experiments, 12 replicates/group. *, p < 0.001 versus pHOGL3/4.5 +220 CEBP-α deletion and wild-type constructs. C, HK-2 cells were transfected with siRNA targeted against Sp1 or mock siRNA as described under “Experimental Procedures.” Cells were allowed to recover for 16 h and then transfected with the pHOGL3/4.5 +220 construct or DMSO controls. Five hours after transfection, cells were split into 12-well trays, and 24 h later, they were then induced with hemin (5 μM). Luciferase activity is presented as means ± S.E., n = 3 independent experiments, 12 replicates/group. *, p < 0.001 versus other groups. D, decoy oligonucleotide targeting indicates that Sp1 consensus sequences are required for enhancer activity. HK-2 cells were transiently cotransfected with pHOGL3/4.5 +220 and double-stranded WT or mutated decoy (Mut decoy) oligonucleotides. Transfected cells were analyzed for luciferase activity. Data are represented as means ± S.E., n = 3 independent experiments. *, p < 0.001 versus other groups. E and F, Sp1 associates with the 220-bp enhancer region by a ChIP assay during hemin stimulation in HK-2 cells. E, sequence of the 220-bp enhancer region with the primers used for the ChIP assay. The Sp1 sequence is underlined. HK-2 cells were treated with 5 μM hemin for 2 h or with vehicle and processed for the ChIP assay with Sp1 antibody as described under “Experimental Procedures.” IgG was used instead of the primary antibody as a negative control. DNA isolated from the immunoprecipitation was amplified with primers (Table 1) for the regions containing the 220-bp intronic enhancer (F), the −4.0 kb HS-2 region (G), and the +2.2 kb region in intron 2 (H). Results shown are signal/noise ratio against input over background from three independent experiments (mean ± S.E.).
Table 1

List of primers used for the 3C and ChIP assays and real-time PCR

| Primer name | Sequence (5’→3’) |
|-------------|-----------------|
| F1          | CTGCCCTCTGCTGAGTTATCTCCTGAGC |
| F2          | GTCATAGCTCGTACGACAAGGAGC |
| F3          | GAAACGACCCGACCGACAGGAC |
| R5          | GCAGCAGTCAAGACAGGACAGG |
| R5N         | GTACGCTAGGACAGGAGACAGG |

To determine if these trans-acting factors interacted with the enhancer region in vivo, we performed ChIP assays and analyzed the interaction of Sp1 with the 220-bp enhancer region, −4.5 kb promoter region, and consensus E-box-containing region located at +2.2 kb of the human HO-1 gene. The sequence of the 220-bp enhancer region with the putative Sp1 binding site is outlined in Fig. 2E, and primers utilized for ChIP RT-PCR are listed in Table 1. As shown in Fig. 2F, Sp1 associated with the 220-bp enhancer region in the presence of hemin, whereas no significant association was observed in the −4.0 and +2.2 kb regions (Fig. 2, G and H).

Because Sp3 can also bind to the Sp1 consensus binding sequences (22–27), ChIP assays were performed in control and hemin-treated HK-2 cells using Sp3 antibodies. Regardless of hemin stimulation, Sp3 was not associated with the 220-bp enhancer, the −4.0 kb promoter, and the +2.2 kb regions (supplemental Fig. 2, A–C). In addition, Sp3 siRNA was utilized to evaluate the involvement of Sp3 in hemin-mediated HO-1 induction. Effective knockdown of Sp3 mRNA and protein was achieved in cells transfected with Sp3 siRNA. However, hemin-induced HO-1 expression (mRNA and protein) was not attenuated by Sp3 siRNA (supplemental Fig. 2, D–F).

Mutations of the HO-1 Promoter and Its Effects on Enhancer Function—In previous work, we had detected multiple DNase I-hypersensitive (HS-1 to -4) regions within the HO-1 promoter and characterized two of these (HS-1 located at −44 bp and HS-2 located at −4.0 bp) by dimethylsulfate in vivo footprinting (18, 19) (Fig. 3A). Three critical elements, namely CRE, a downstream NF-E2/ AP-1 element (ARE-5) (located at the −4.0 kb HS-2 region), and an E-box sequence (M3 in Fig. 3; located at the −44 bp HS-1 region) were detected and required for hemin-inducible promoter activation (18, 19). In view of the earlier finding that promoter sequences were required for enhancer function, site-directed mutagenesis of these three critical elements (CRE, ARE-5, and E box) of the promoter was performed using the pHOGL3/4.5+220 construct. The wild-type and mutated sequences are shown in Fig. 3A. Mutations were verified by sequencing, and transfections were performed in HK-2 cells. Analysis of enhancer activity following transfection of these pHOGL3/4.5+220 mutated constructs indicated the

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![Diagram of HO-1 promoter and enhancer interactions](image-url)

**Figure 3. Mutations in the human HO-1 promoter attenuate enhancer activity in HK-2 cells.** A, schematic showing the −4.5 kb human HO-1 promoter with exons 1 (E1) and 2 (E2), including the 220-bp enhancer in intron 1. The regulatory elements in the −4.0 kb HS-2 region (containing the CREB and AP-1 sites) and the −44 bp HS-1 region (containing the E box region, which binds to USF-1/2) with the wild-type sequences and mutated sequences are shown. P, Pest; X, Xbal. ΔCRE, deletion of the CRE; the E-box M3 and the ARE-5 point mutations are indicated by the lowercase t. B, cells were transfected with the indicated plasmid constructs containing mutations in the HO-1 promoter (ΔE box, CRE, and ARE-5 or combination of ARE-S + E box deletion and deletion of CRE + ARE-5 mutation). Mutations or deletion of the regulatory regions in the promoter are indicated by X. Luciferase activity was determined following stimulation with vehicle (DMSO) or hemin (5 μM). Values are mean S.E. (error bars), n = 2 independent experiments with 12 replicates/group. *p < 0.001 versus all other groups by analysis of variance.
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FIGURE 4. Time-dependent chromatin looping between the −4.0 kb promoter region and the 220-bp intronic enhancer region by the 3C assay. A, HK-2 cells were treated with 5 μM hemin or vehicle (DMSO) for 2 h, and the 3C assay was performed as described under “Experimental Procedures.” 300 ng of the sample was amplified by PCR. A human bacterial artificial chromosome (HO-1 BAC; GenBank accession number Z82244) clone was used as a positive control, and non-cross-linked genomic DNA from HK-2 cells (HK-2 DNA) was used as a negative control. Reverse-cross-linked DNA was then amplified with primers (Table 1 and schematic in D). PCR products were electrophoresed in a 1% agarose gel. B, extracted DNA was amplified with primers F3 and R1 by real-time PCR to quantitate the cross-linking efficiency between the fragment containing the −4.0 kb promoter and 220-bp enhancer region. Relative Ct values from each sample were normalized with products containing BAC DNA was used as a positive control, and genomic DNA obtained from HK-2 cells without cross-linking was used as a negative control in these experiments (Fig. 4A). Real-time PCR data demonstrated that hemin-treated samples had cross-linking efficiency that was significantly higher than that of vehicle-treated samples (Fig. 4B) with the F3/R1 primer pairs. These results suggest that the two DNA fragments, one containing the regulatory promoter sequences at −4.0 kb region and the other with the 220-bp intronic enhancer, are potentially interacting or are in close proximity with each other. The other primer pairs specific for the DNase I-hypersensitive site HS-3 (located at −7.2 kb) (F2/R1) and HS-4 (located at −9.2 kb) (F1/R1) identified previously (18) were not detected, indicating that the enhancer-containing DNA fragment was not in close proximity with the HS-3 or HS-4 region. HO-1-containing BAC DNA was used as a positive control, and genomic DNA obtained from HK-2 cells without cross-linking was used as a negative control in these experiments (Fig. 4A).

To determine the time course of the DNA looping interaction between HS-2 and the internal enhancer, HK-2 cells were treated with hemin (5 μM) for the indicated times, and 3C assays were performed with quantitative real-time PCR using the F3/R1 primer pair (Fig. 4C). The results demonstrated that the interaction between these two regions of chromatin reached a peak after 2 h of hemin stimulation and started to diminish thereafter, declining to nearly basal levels by 16 h, consistent with the time course of endogenous HO-1 mRNA induction by exposure to hemin in renal epithelial cells (15). To confirm the 3C data and validate the assay, the PCR product generated by the F3/R1 primer pair was extracted, requirement of these regulatory elements within the promoter for both basal and hemin-mediated activation of the enhancer (Fig. 3B). These results also suggested a potential interaction of regulatory elements within the HO-1 promoter and the minimal intronic enhancer region.
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TA-cloned, and sequenced. The resulting DNA sequence contains portions of both the DNA fragments from the HS-2 region and the 220-bp enhancer region, ligated by the BglII restriction enzyme cleavage site (Fig. 4D). This restriction site could only be generated after the ligation step during the 3C assay and again confirms the DNA looping interaction between these two distal regions. The PCR products observed in the vehicle-treated samples (Fig. 4A) were also TA-cloned and sequenced and found to be nonspecific.

3C Assay Using Two Restriction Enzymes Confirmed the DNA Looping Interactions—The DNA looping detected by the 3C assay was further validated using an additional restriction enzyme ApaLI along with BglII. The 220-bp enhancer region has a recognition site for ApaLI, and restriction digestion with both ApaLI and BglII would result in an additional fragment with BglII at one end and ApaLI at the other end (Fig. 5A). If this additional fragment of DNA is not cross-linked with associated proteins or DNA, a PCR product would not be generated by the primers F3/R1 because the DNA fragment generated by ApaLI and BglII would not be compatible for the ligation. To test this, the 3C assay was performed with BglII and/or ApaLI on HK-2 cells with or without hemin induction. When only BglII was used to digest DNA during the 3C assay, the expected PCR product from the F3/R1 primer pair was obtained following hemin induction (Fig. 5B), which was confirmed by sequencing. However, when both BglII and ApaLI were used, using the same primer pairs, no PCR product was obtained after hemin induction (Fig. 5B). Quantitative real-time PCR was performed to measure and compare the relative cross-linking efficiency when BglII alone or ApaLI and BglII were used together. Amplified product was not detected from the sample in which both restriction enzymes were used (Fig. 5C) but was present with BglII alone. These results suggest that chromatin looping mediates the interaction of regulatory elements within the HO-1 promoter and the 220-bp enhancer.

Sp1 Regulates the DNA Looping Interactions between the Intrinsic Enhancer and the Distal HO-1 Promoter—Because ChIP and Sp1 siRNA knockdown analyses indicated an important role for the transcription factor, Sp1, for hemin-mediated enhancer function, we determined whether Sp1 siRNA knockdown would attenuate chromatin looping between the intrinsic enhancer and the promoter regions. Transfection with Sp1 siRNA in HEK293 cells resulted in successful knockdown of Sp1 protein and mRNA levels, respectively, compared with mock siRNA or the control (transfection reagent alone) (Fig. 6, A and B). HEK293 cells were used in these experiments due to their higher level of transfection efficiency. 3C assay and quantitative real-time PCR following Sp1 siRNA-mediated knockdown of Sp1 resulted in a loss of interaction between the intrinsic enhancer and the HS-2 region of the distal HO-1 promoter following stimulation with hemin as indicated by a marked decrease in cross-linking efficiency for the F3/R1 primer pair combination (Fig. 6C). We further investigated the role of Sp1 using mithramycin A, a GC binding inhibitor of Sp1. Hemin-mediated increase in endogenous HO-1 protein expression was inhibited in the presence of nanomolar concentrations of mithramycin (Fig. 6D) in HEK293 cells. Additionally, 3C assays in the presence of mithramycin A significantly reduced the relative cross-linking efficiency using the F3/R1 primer pairs, suggesting the loss of interaction between the HS-2 region and the intrinsic enhancer (Fig. 6E). These results demonstrate the critical role of Sp1 for stimulus-dependent chromatin looping between the intrinsic enhancer and the HO-1 promoter region.

DISCUSSION

One of the critical requirements for normal cell function and survival is the ability to surpass changes in the environment and to mount a positive response against stress. It is well documented that HO-1 can be induced as an adaptive response by a variety of oxidant stimuli, such as heme, heavy metals, growth factors, cytokines, modified lipids, and others (reviewed in Ref. 13). The products of HO-mediated heme degradation have also been directly linked with the regulation of important biological processes, including oxidative stress, inflammation, apoptosis, cell proliferation, angiogenesis, and fibrosis (2, 3, 28–33). Although the downstream effector pathways for the products of HO activity have been studied, the complex and intricate mechanisms utilized by the components of the gene-regulatory machinery to promote chromatin accessibility of the human HO-1 gene have not been fully investigated.

Several key findings that provide new mechanistic insights for the regulation of human HO-1 gene expression are reported in this work. First, we have characterized a 220-bp region within intron 1 of the human HO-1 gene that functions as a minimal enhancer and regulates hemin-mediated human HO-1 gene expression via interaction with the distal HO-1 promoter. Second, we demonstrate the functional significance of an Sp1 binding site in the minimal enhancer region that is required for
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FIGURE 6. Sp1 is required for chromatin looping between the HS-2 region of the distal HO-1 promoter and the intronic enhancer. Shown are Sp1 protein (A) and mRNA (B) analyses in HEK293 cells following transfection with oligofectamine (control) alone, mock siRNA, or Sp1 siRNA. C, schematic of real-time PCR primers F3 and R1 relative to the HS-2 region and the 220-bp enhancer used for 3C assay (top). B, BglII 3C assay performed on HEK293 cells stimulated with 5 μM hemin following transfection with oligofectamine alone, mock siRNA, or Sp1 siRNA. Quantitative real-time PCR was performed using F3 and R1 primers showing reduction in cross-linking efficiency with Sp1 siRNA. Results are shown as -fold increase from three independent experiments performed in triplicate each time (mean ± S.E. error bars). *, p < 0.05. D, Western blot showing reduction in hemin-mediated increase in HO-1 protein expression in HEK293 cells following treatment with 10 and 100 nM mithramycin A, a GC binding inhibitor of Sp1. Actin levels were used as loading controls. E, 3C assay performed on HEK293 cells in the presence or absence of 100 nM mithramycin A and stimulated with control (vehicle) or 10 nM hemin. Quantitative real-time PCR using F3 and R1 primers was performed to measure the cross-linking efficiency after the 3C assay. Results are shown as -fold increase from three independent experiments performed in triplicate each time (mean ± S.E.). *, p < 0.05.

Sp3 does not associate with this consensus sequence and is not required for hemin-mediated HO-1 induction. Third, we show that at least three important regulatory elements within the −4.0 kb region of the human HO-1 promoter are necessary for enhancer activity, suggesting potential interaction between the internal enhancer and distal HO-1 promoter regions. Finally, using the 3C assay, we present in vivo evidence for chromatin looping between the HO-1 promoter and intron enhancer (located −6.0 kb apart) and the requirement of the transcription factor, Sp1, for this molecular interaction.

Intrachromosomal interactions resulting in chromatin looping have been shown to be involved in promoting transcriptional activation of various genes in eukaryotes. The 3C assay has provided a mechanism to study such changes in chromatin conformation that bring distant regulatory regions spanning >50–100 kb in close proximity, contributing to gene activation (34, 35). For example, using the 3C assay, looping between the β-globin locus control region and the β-globin promoter located ∼50 kb apart has been demonstrated (36). Long range looping by the 3C assay between distant regulatory elements has also been documented for other genes, including the C-reactive protein (37), the peptidylarginine deamidase 3 (38, 39), and interferon γ genes (40). More recently, similar to our results with chromatin looping for the HO-1 gene, DNA looping over short ranges (∼100 bp to 10 kb) have been described for the murine-inducible nitric-oxide synthase (41, 42), human CD68 (43), insulin (44), and the neonatal Fc receptor (FcRn) for IgG genes (45). In the case of the FcRn gene, DNA looping interactions were reported between intronic NF-κB sequences and the FcRn promoter spanning over a ∼12-kb distance (45). Whether similar interactions between promoters and enhancers occur for other genes that have intronic regulatory sequences, such as manganese superoxide dismutase (46), platelet-derived growth factor-A (46), alcohol dehydrogenase-1 (47), and aquaporin 5 (48), remains to be confirmed by the 3C assay.

Previously, we have demonstrated that there are four Dnase I HS sites within the human HO-1 promoter located at −44 bp (HS-1), −4.0 kb (HS-2), −7.2 kb (HS-3), and −9.2 kb (HS-4) (18). In vivo footprinting of the HS-1 and HS-2 sites revealed multiple protective guanine nucleotides that were required for HO-1 gene expression (18, 19). In this work, we have thus far defined the interactions between the HS-2 region in the promoter with the intronic enhancer following hemin stimulation. However, multiple regions within the HO-1 promoter seem to be interacting with each other. In addition, the results with the deletion of the 4.0-kb region at the 3′-end of the +12.5 kb enhancer (Fig. 1B) suggest the presence of additional regulatory elements in this region interacting with the promoter and are currently being explored in more detail.

Our previous studies identified members of the Jun protein family (JunB, c-Jun, and JunD) and USF-1/2 (upstream stimu-
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