Inhibition of Tartrate-Resistant Acid Phosphatase Gene Expression by Hemin and Protoporphyrin IX. Identification of a Hemin-Responsive Inhibitor of Transcription

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Tartrate-resistant acid phosphatase (TRAP) is an iron-containing protein encoded by the same gene that codes for uteroferrin, a placental iron transport protein. In human peripheral mononuclear cells, TRAP expression is inhibited by both hemin (ferric protoporphyrin IX) and protoporphyrin IX. Nuclear run-on assays confirmed that this inhibition occurs at the level of gene transcription. Previous studies with mTRAP deletion mutants showed that the hemin effect was dependent on repressor activity in the mTRAP 5' flanking region at -1846 bp to -1240 bp relative to ATG (Reddy et al, J Bone Mineral Res 10:801, 1995). We now report that gel shift assays showed a DNA binding protein in nuclear extracts of hemin-treated cells termed hemin response element binding protein (HREBP). Additional studies have localized the HREBP binding region in the mTRAP 5' flanking DNA to a 27-bp sequence at -1815 to -1789 bp relative to ATG. A tandem repeat sequence, GAGGC, is present within this DNA segment, shown to be involved in binding of HREBP. Highly homologous sequences are present in the 5' flanking region of the hTRAP gene. Binding of HREBP to the mTRAP DNA sequence was inhibited by anti-HAP1 antibodies, indicating homology between the hemin-responsive factor and the yeast heme-dependent transcription factor, HAP1. A 607-bp segment of the mTRAP 5' flanking region containing the candidate hemin response element and surrounding sequences conferred hemin regulation on the viral SV40 promoter. Southwestern blotting experiments probing nuclear extracts of hemin-treated U937 cells with the 27-bp binding sequence showed two protein bands at 37 and 133 kD representing candidate HREBPs. A GENINFO search showed several other mammalian genes with tandem GAGGC motifs in noncoding regions, providing the possibility that additional genes may also be regulated by hemin at the level of transcription. These studies provide the first description of a novel iron/hemin-responsive transcriptional regulatory mechanism in mammalian cells.

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MATERIALS AND METHODS

Preparation of human peripheral mononuclear cell (PMC) populations and cell cultures. PMCs were isolated from the venous blood of healthy volunteers by density sedimentation on ficoll-Hyphaque. To assess TRAP expression, PMCs were incubated for various times at 37°C in 5% CO₂ in RPMI medium containing 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) without any additives or 50 μM hemin or protoporphyrin IX.

RNA hybridization analyses. Total cellular RNA was isolated by a modification of the guanidinium isothiocyanate technique. Integrity of RNA was assessed by visualization of ethidium bromide-stained gels. Total cellular RNA was fractionated by electrophoresis through a 1% (wt/vol) agarose gel containing formaldehyde and then transferred to nitrocellulose filters presoaked in 20× SSC (SSC = 0.15 mol/L NaCl, 15 mmol/L sodium citrate dihydrate). The filters
were hybridized with 32P-oligolabeled cDNA probes. After washing, the filters were exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) at -70°C with Dupont Cronex intensifying screens (Du- pont, Wilmington, DE). Relative intensities of bands on autoradiography were quantified by scanning densitometry.

Three cDNA probes were used: a full-length human placental TRAP cDNA probe of 1,412 bp; a 3.8-kb EcoRI-HindIII fragment from the human α-actin genomic clone, H4A4;12 and a 2.2-kb cathep- sin D cDNA probe containing the procathepsin D coding sequence.13

**Nuclear transcription assay.** Active nuclei from human PMCs were isolated as described by Marzluff and Huang4 using lysis buffer consisting of 0.32 mol/L sucrose in 3 mmol/L CaCl2, 2 mmol/L magnesium acetate, 0.1 mmol/L EDTA, 0.1% Triton-X-100, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 10 mmol/L Tris-HCl, pH 8.0. After washing, nuclei were resuspended in 25% glycerol, 5 mmol/L magnesium acetate, 0.1 mmol/L EDTA, 5 mmol/L dithiothreitol, 50 mmol/L Tris-HCl, pH 8.0, at a concentration of 2 × 106 nuclei/mL. Nuclear transcription was performed by a modification of the procedures of Marzluff and Huang14 and Evans et al.15 Run-on transcription was performed for 45 minutes at 25°C in an assay mixture of 0.5 mmol/L each of ATP, CTP, and GTP; 0.25 to 0.5 mmol/L [α-32P]UTP (500 to 1,000 μCi/mL); 0.25 mmol/L S-adenosylmethionine; 0.12 mol/L KCl; 5 mmol/L magnesium acetate; 12.5% glycerol; 0.05 mmol/L EDTA; 2.5 mmol/L dithiothreitol; and 25 mmol/L Tris-HCl, pH 8.0. mRNA was purified by sequential treatment with DNase and proteinase K and then extracted with phenol/chloroform (1:1). The final RNA product was precipitated overnight in 2 vol of ethanol and recovered after two washes in 70% ethanol. Labeled RNA (10 to 20 × 106 cpm) was hybridized to nitrocellulose filters containing 5 μg of immobilized DNA in halving dilutions. Filters were prehybridized for 1 hour at 45°C and hybridized with RNA for 3 days at 45°C in 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 50 μg/mL tRNA, 6X SSC, 0.1% NaP104, 2X Denhardt’s solution without bovine serum albumin (BSA), and 10% dextran sulfate. Filters were washed in three changes of 2× SSC, 0.1% NaP104, and 0.1% SDS for 10 minutes at 25°C. A high stringency wash then was performed at 65°C in two changes of 0.2% SSC, 0.1% SDS for 10 minutes each. Filters were air-dried for 30 minutes and then autoradiography was performed as described above. In preliminary experiments, α-amanitin (2 μg/mL) inhibited transcription of genes for TRAP and cathepsin D, but not of the gene for 28S RNA.

**Gel retardation assays.** We used the technique of Andrews and Faller4 to extract DNA-binding proteins. Briefly, HRE H9 or U937 cells (5 × 106 to 1 × 107) were suspended in 400 μL of cold 10 mmol/mL HEPES-KOH, pH 7.9, 1.5 mmol/mL MgCl2, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, and 0.2 mmol/L PMSF for a 10-minute swelling period at 4°C. After vortexing and centrifugation to release and pellet the nuclei, the pellet was suspended in 20 to 100 μL of cold 20 mmol/mL HEPES-KOH, pH 7.9, 25% glycerol, 420 mmol/mL NaCl, 1.5 mmol/mL MgCl2, 0.2 mmol/L EDTA, 0.5 mmol/L dithio- threitol, and 0.2 mmol/L PMSF and extracted for 20 minutes at 4°C. After centrifugation at 4°C to remove debris, the supernatant fraction containing DNA binding proteins was recovered. DNA probes (10 mmol/mL) were radiolabeled with 10 mCi/mL γ-32P (specific activity, 1,000 Ci/mmol) by reaction with [γ-32P]ATP and T4 polynucleotide kinase for 30 minutes at 37°C. The reaction was stopped with 0.5 mmol/L EDTA, and probes were purified by phenol/chloroform extraction and ethanol precipitation. Probes were suspended in TE buffer and used in binding reactions (10,000 to 20,000 cpm) with 2 to 10 μg nuclear extract protein incubated at 37°C for 30 minutes with BSA and poly-(dI-dC). After incubation, reaction mixtures were analyzed by electrophoresis in a 4% low ionic strength native polyacrylamide gel with an acrylamide: bisacrylamide ratio of 80:1 followed by auto- radiography.

**Oligonucleotide-protein hybridization (Southern blots).** Nuclear extract (10 to 30 μg) was mixed 1:1 with 5% NaDodSO4, 5 mmol/L Tris-Cl, pH 8.0, 8.0 mmol/L dithiothreitol, 20% glycerol, and 0.05% pyronin Y. Samples were electrophoresed in 7% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 4°C without prior boiling. Proteins were transferred electrophoretically to support nitrocellulose in 25 mmol/L Tris and 190 mmol/L glycine. For binding, nitrocellulose membranes were incubated at 27°C for 10 minutes twice in 25 mmol/L HEPES-KOH, pH 7.6, 12.5 mmol/L MgCl2, 20% glycerol, 0.1% NP-40, 0.1 mol/L KCl, 1 mmol/L dithiothreitol, 10 μmol/L ZnSO4, and 6 mol/L guanidine HCl. After incubation, the transferred proteins were gradually renatured by subsequent 10-minute incubations in halving dilutions of the 6 mol/L guanidine HCl buffer until the guanidine HCl concentration was 0.188 mol/L. Filters were then incubated for 10 minutes in buffer without guanidine HCl, followed by 30 minutes in 5% (vol/vol) nonfat dry milk in 10 mmol/L HEPES, pH 8.0. Filters next were washed for 5 minutes with 10 mmol/L HEPES, pH 8.0, 50 mmol/L NaCl, 10 mmol/L MgCl2, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 0.25% nonfat dry milk, followed by the addition of fresh buffer containing 10 5 cpnm/mL 32P-labeled oligonucleotide probe. After incubation for 30 minutes at 27°C, filters were washed three times with 15 minutes in 25 mmol/L HEPES-KOH, pH 7.6, 12.5 mmol/L MgCl2, 20% glycerol, 0.1% NP-40, 0.1 mol/L KCl, 1 mmol/L dithiothreitol, and 10 μmol/L ZnSO4. Filters were visualized by autoradiography.

**Transfection assays.** The mTRAP-luciferase fusion gene (pKBS) was constructed as previously described by insertion of mTRAP sequences from the region −1846 to +2 bp into the Km U68I II sites of a promoter-deficient pGL2 basic vector containing the luciferase reporter gene.16 The rabbit endometrial cell line, HRE H9, was used for transfection experiments because it is known to be permissive for mTRAP promoter constructs, and mouse cell lines permissive for TRAP expression have not been described. We have previously shown the transcriptional regulation of mTRAP promoter activity by hemin and iron in HRE H9 cells transiently transfected with luciferase reporter gene constructs.17 HRE H9 cells were plated at a density of 2 × 106 cells/100-mm dish in a Minimal Essential Medium with 10% FBS 24 hours before transfection using the CaPO4 method (Stratagene, La Jolla, CA). For transfections, 15 μg of cesium chloride gradient-purified plasmids was used. Cotransfection with pKSV β-galactosi- dase was performed as a control for transfection efficiency.20 To develop stably transfected cell lines, the neomycin-resistant plasmid PWL neo (Stratagene) was cotransfected with TRAP-luciferase plasmids at a 1:3 molar ratio. Cells were exposed for 16 hours to the CaPO4/DNA precipitate, washed twice with serum-free medium, fed with fresh culture medium supplemented with 10% FBS, and then incubated for 48 hours at 37°C in 5% CO2. G418 treatment (400 μg/mL) was applied 48 hours after the removal of DNA from the cultures and was maintained for 30 days before beginning the experiments. Stable clones bearing constructs of interest were derived by limiting dilution.

To test the response to hemin, transfectants were incubated for 48 hours with or without hemin (50 μmol/L). After incubation, the cell monolayer was washed twice with phosphate-buffered saline and incubated at room temperature for 15 minutes with 0.3 mL cell- lysis reagent (25 mmol/L Tris, pH 7.8, 2 mmol/L dithiothreitol, 0.2 mmol/L 1,2-diaminoethanesulfonic acid-Na2, 10% [vol/vol] glycerol, and 1% [vol/vol] Triton X-100). The monolayer was scraped and spun briefly in a microcentrifuge to pellet the debris. Luciferase activity was assayed in the supernatant by mixing a 20-μL aliquot with 100 μL of luciferase assay reagent (20 mmol/L

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Fig 1. Inhibition of TRAP expression by hemin. Human PMCs were cultured in RPMI medium containing 10% heat-inactivated FBS for 48 hours with or without hemin (50 μmol/L). Cells then were harvested. Total RNA was extracted and separated by formamide agarose gel electrophoresis and transferred to nitrocellulose filters. The filters were hybridized with 32P-oligolabeled cDNA probes for TRAP (A) or α-actin (B). Lane 1, time 0; lane 2, 48 hours, no hemin; lane 3, 48 hours + hemin (50 μmol/L).

Effect of hemin on cells transfected with mTRAP/lucifer-

tricine, 1.07 mmol/L MgCO3, 2.67 mmol/L MgSO4, 0.1 mmol/L EDTA, 33.3 mmol/L dithiothreitol, 270 μmol/L coenzyme A, 470 μmol/L luciferin, and 530 μmol/L ATP). Light emission was measured for 20 seconds of integrated time using a Turner TD-20e Luminometer and following the manufacturer’s instructions (Promega, Madison, WI).

RESULTS

Effect of hemin or protoporphyrin IX on expression of TRAP mRNA in PMC cultures. Figure 1 shows a representative experiment examining the appearance of TRAP mRNA transcripts in cultures of human PMCs incubated with or without the addition of hemin (50 μmol/L). The marked induction of TRAP mRNA transcripts seen after 48 hours in control cultures was blocked by the addition of hemin. We have previously shown that induction of TRAP mRNA and enzyme activity in these cultures is localized to the monocyte-macrophage component of the PMC population.7 Figure 2 shows the results of an identical experiment performed with protoporphyrin IX or cobalt protoporphyrin IX in place of hemin. Inhibition of TRAP mRNA expression again was observed. Cell viability, as judged by trypan blue exclusion, was not different among cultures with hemin, protoporphyrin IX, cobalt protoporphyrin, or controls and always exceeded 95% at 48 hours. To determine whether the TRAP mRNA induction observed in the PMC cultures was transcriptionally mediated, we performed nuclear run-on assays using nuclei isolated from untreated or hemin-treated PMCs (Fig 3). The results of these experiments indicated that both the induction and also the hemin inhibition of TRAP mRNA expression in PMCs in vitro are mediated at the level of gene transcription.

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Fig 2. Inhibition of TRAP expression by protoporphyrin IX. PMCs were cultured, and TRAP expression was analyzed as described in the Fig 1 legend. (A) TRAP probe: Lane 1, 48 hours, no protoporphyrin IX; lane 2, time 0; lanes 3 and 4, 48 hours + protoporphyrin IX (50 μmol/L). (B) Actin probe: Lanes as in (A). (Lower panel) TRAP probe: Lane 1, 48 hours; lane 2, time 0; lane 3, 48 hours + hemin (50 μmol/L); lane 4, 48 hours + cobalt protoporphyrin (50 μmol/L).

Fig 3. Nuclear transcription assays. Nuclei from human PMCs were isolated then incubated with radiolabeled nucleotides as described in Materials and Methods. mRNA extracts (equal counts per minute) were hybridized to nitrocellulose filters precoated with human placental TRAP cDNA or cathepsin D cDNA. Filters were autoradiographed to visualize dots. Lane 1, time 0; lane 2, 48 hours untreated; lane 3, 48 hours + hemin. Each lane depicts four halving dilutions of immobilized cDNA. (A) TRAP cDNA; (B) cathepsin D cDNA.
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Fig 4. (A) Inhibition of luciferase expression by hemin in HRE H9 cells stably transfected with mTRAP-luciferase chimeric constructs. Stable transfectants were produced and then cultured with or without hemin for 48 hours. Cell lysates then were assayed for luciferase activity. Luciferase activity was inhibited by hemin in pKB5 transfectants (mTRAP -1846 to +2 bp), but not in pkSSSt1 transfectants (mTRAP -330 to +2 bp). Inhibition in pKB5 is blocked by excess 27-mer corresponding to mTRAP -1815 to -1789 bp relative to ATG, a candidate binding site for a hemin-responsive nuclear factor, but not by an irrelevant oligo. In these experiments, pKB5 transfectants were exposed to 5 μmol/L concentrations of the 27-bp binding site oligo or an irrelevant oligo for 12 hours preceding and for 48 hours during treatment with hemin (50 μmol/L). Fresh oligonucleotides were added to the cultures every 12 hours during the incubation period. (B) Effect of time of hemin addition on TRAP expression by human PMCs. On the left are Northern blots of RNA extracted from PMCs cultured as follows: lane 1, untreated for 48 hours; lane 2, untreated x 24 hours, hemin added for 24 to 48 hours. (A) TRAP; (B) α-actin. On the right are densitometric scans of the data in (A).

ase gene constructs. To examine the effect of hemin on TRAP transcription in more detail, we prepared promoter/reporter mTRAP/luciferase gene constructs and performed transfection experiments into the HRE H9 cell line. This cell line derived from rabbit endometrium was chosen because it has been used by us and others to study TRAP regulation.7,8,19 Based on these previous data, HRE H9 cells were known to be capable of expressing and regulating transfected TRAP genes, although they do not express endogenous TRAP. The 5′-flanking region of the mTRAP gene has promoter activity that is responsive to iron and hemin.7,8 Incubation with hemin of stable HRE H9 transfectants bearing a full-length (−1846 to +2 bp) mTRAP promoter linked to a luciferase reporter inhibited luciferase activity by greater than 50% (Fig 4A). By contrast, transfectants bearing promoter/reporter constructs with only −330 to +2 bp of the mTRAP 5′-flanking region were not inhibited by hemin. The hemin inhibition was blocked by incubation with excess 27-bp oligonucleotides derived from the mTRAP 5′-flanking region (discussed in detail below) but not by irrelevant oligos (Fig 4A).

In the transfection experiments (Fig 4), inhibition by hemin was less than that seen in the primary PMC culture system (Fig 1). A basic difference between the transfected cells and primary culture system is that there is a constitutive baseline level of luciferase activity driven by the mTRAP promoter in the stable transfectants, whereas, in the human PMCs, the TRAP gene is not expressed under baseline conditions. We considered the possibility that hemin inhibition might be more effective in blocking induction of TRAP expression than suppressing transcription once the gene has become transcriptionally active. Therefore, we performed the experiments shown in Fig 4B. In these experiments, hemin was added to PMC cultures after 24 hours of incubation rather than at the time of culture initiation, as in Fig 1. The results show that hemin, added to cultures after 24 hours of incubation, can blunt but not block completely the level of TRAP mRNA in PMC cultures. In fact, the level of inhibition in these experiments (60% to 70%) closely approximates the inhibition seen in the transfection experiments in Fig 4A (approximately 50% to 60%). These results are consistent
with the interpretation that hemin is most effective at blocking induction of gene expression. One must also consider that residual mRNA from the initial induction period may partially contribute to the results seen in Fig 4B. And, we cannot exclude the possibility that additional hemin-responsive elements may be located elsewhere in the mTRAP gene. Nonetheless, taken together, the data in Fig 4 suggest that the mTRAP 5'-flanking region contains specific sequences that mediate hemin inhibition of mTRAP expression.

Gel retardation experiments. To search for hemin-responsive DNA binding proteins that might mediate transcriptional inhibition of TRAP expression, we performed gel retardation assays using nuclear extracts from control and hemin-treated cells. Initially HRE H9 nuclei were used to document the presence of such proteins in cells that we knew regulated TRAP expression.

However, we were interested in studying this regulation in human cells. We have recently cloned the human TRAP gene, and Fleckenstein et al have elucidated the genomic structure of hTRAP. Sequencing of the 5'-flanking region to −3026 bp relative to ATG showed two separate GAGGC repeats separated in one instance by 1 bp and in the other by 3 bp. These two areas are homologous to the mTRAP candidate hemin response element described below and may represent the analogous human element. Based on these observations, we have used nuclear extracts from human U937 monocytic cells with mTRAP oligonucleotide probes for the gel retardation assays described below. As with HRE H9 cells, U937 cells do not possess endogenous TRAP, but they do possess hemin-responsive DNA binding proteins, and they are derived from the monocyte lineage that is known to express TRAP in vivo.

We used a strategy of deletion mutagenesis to identify a candidate hemin response element in the mTRAP 5'-flanking region. Initial gel shift assays showed protein binding to a 254-bp probe corresponding to −1843 to −1590 bp of the mTRAP 5'-flanking region after incubation with nuclear extracts from hemin-treated U937 cells. Controls to confirm the specificity of this observation included competition experiments with excess unlabeled relevant and irrelevant oligos and gel shift experiments with radiolabeled irrelevant oligos. Also, simple addition of hemin to nuclear extracts from untreated cells did not reproduce the gel shifts. These data indicated that a DNA binding site(s) for a potential transcriptional repressor(s) was contained within this segment. The data also suggested that incubation of cells with hemin was required for induction or activation of this factor.

Because of the potential for binding of many different proteins to a large labeled probe, additional experiments were performed using shorter probes from the −1843 to −1590 bp region. This permitted localization of a binding region to a 27-bp sequence corresponding to bp −1815 to −1789 bp. Examination of this 27-bp probe shows the sequence: ACCTTGAGGCCAGGCAGGTAAATGG. A tandem repeat sequence is highlighted. Gel shift assays with this probe are shown in Fig 5. Whereas nuclear extracts from untreated U937 cells showed baseline binding to the 27-bp probe, extracts from hemin-treated cells exhibited a clear gel shift pattern with increased binding of at least one higher molecular weight protein to the radiolabeled probe (Fig 5A). Binding was blocked by excess cold relevant but not irrelevant probe. Figure 5B shows that similar DNA binding proteins are present in human PMCs. These data provide evidence that a DNA binding site for a nuclear protein termed hemin response element binding protein (HREBP) is present at this location in the mTRAP 5' flanking region. Additional experiments were performed to confirm the specificity of this interaction (Fig 6). In these experiments, 20-fold molar excess of different oligonucleotides derived from the 27-bp sequence were used as cold competitors in gel mobility assays. The results indicate that GAGGC completely abolished and GCAGG partially inhibited the observed gel shift. By contrast, no to minimal inhibition was given by either CCTTG or TAATG. These experiments provide strong evidence that the GAGGC tandem repeat at −1809 to −1800 bp of the mTRAP 5' flanking region is involved in the binding of a nuclear factor (HREBP) expressed in hemin treated cells. However, sequences in addition to the tandem GAGGC
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**Fig 6.** Specificity of HREBP binding to the mTRAP promoter. Gel mobility assays were performed as described in the Materials and Methods with U937 cell extracts and radiolabeled 27-bp mTRAP 5′-flanking region (−1815 to −1789 bp) probe. In lanes 4 through 11, a molar excess (20-fold) of the designated unlabeled oligonucleotide competitors was added. FP, free probe; C, control cells; H, hemin-treated cells. Lanes 2 and 3, no competitor; lanes 4 and 5, GAGGC competitor; lanes 6 and 7, GCAGG competitor; lanes 8 and 9, TAATG competitor; lanes 10 and 11, CCTTG competitor. The arrow designates the shift band in hemin-treated cell extracts.

Repeat appear to be also required for binding of HREBP to the 27-bp binding site oligonucleotide.

**Inhibition of HREBP binding by anti-HAP1 antibody.** In yeast (*S. cerevisiae*), transcription of a number of genes in the respiratory chain is regulated by heme.9 In the yeast, heme activation of gene transcription is mediated by a nuclear DNA binding protein, HAP1.9 We tested the effect of anti-HAP1 antibody24 (kindly provided by Dr L. Zhang, Massachusetts Institute of Technology, Cambridge, MA) on interaction of HREBP with radiolabeled 27-bp mTRAP binding site DNA. The results are shown in Fig 7. Rabbit polyclonal antibody to the zinc finger domain of HAP1 inhibited binding of HREBP to the 27-bp probe, whereas nonimmune rabbit serum did not. These data suggest that the heme-dependent nuclear factor (HREBP) may share homology with HAP1. Because the gel shift results showed inhibition and not a supershift, it is suggested that both the antibody interaction and the homology are present in the DNA binding domains of HREBP and HAP1.

**Identification of a hemin-responsive nuclear DNA binding protein by Southwestern blotting.** Figure 8 depicts results of Southwestern blotting experiments in which protein blots representing nuclear extracts from hemin-treated U937 cells separated by SDS-PAGE were probed with radiolabeled 27-bp binding site oligonucleotides from the mTRAP 5′-flanking region. The results delineate protein bands of apparent molecular weights of 133 and 37 Kd. Both bands were decreased by excess unlabeled relevant but not irrelevant probe.
Rabbit polyclonal anti-HAP1 antibody blocked delineation of the 133-Kd protein by the 27-bp probe, whereas nonimmune rabbit serum did not. Figure 8 also shows that both the 133- and 37-Kd proteins are present in normal human PMCs after hemin treatment. The lower panel of Fig 8 shows that binding of radiolabeled native 27-bp probe to the 133-Kd protein was not blocked by excess cold probe identical to the native 27-mer except for containing a scrambled GAGGCAGGC sequence. This observation points both to the specificity of binding and also to the importance of the tandem GAGGC sequences to the interaction.

Functional studies of the mTRAP DNA binding site for a hemin-responsive protein. Heterologous promoter experiments were performed to show the functional significance of the 27-bp putative hemin response element (Fig 9). In those experiments, we cloned a 607-bp segment of the mTRAP 5'-flanking region containing the 27-bp candidate binding site (−1846 to −1240 bp) or an irrelevant 626-bp segment (+1349 to +1975 bp relative to ATG) upstream of the SV40 viral promoter in the pGL2 promoter vector. Transient transfections into HRE H9 cells were performed with cotransfection of pRSV β-galactosidase serving as control for transfection efficiency. Hemin treatment caused significant inhibition of luciferase activity in transfectants with the relevant construct, but not in those with the irrelevant construct (Fig 9). This level of inhibition averaged 60%, which is the same as that produced by hemin in the promoter/reporter transfection experiments (Fig 4). Based on our previous studies, we know that this portion of the mTRAP promoter has no endogenous promoter activity, but does contain iron- and hemin-responsive elements. Although consistent with this interpretation, these experiments do not conclusively indicate that the 27-bp binding site is the important sequence for mediating hemin inhibition. Therefore, we have performed blocking experiments with the 27-mer candidate binding motif, as shown in Fig 4. These experiments show that hemin inhibition of luciferase activity in stable transfectants with promoter/luciferase constructs was specifically blocked by incubation with excess 27-mer, but not by an irrelevant oligonucleotide. This observation provides additional evidence that this sequence and its binding protein are involved in hemin inhibition.

**DISCUSSION**

These experiments provide identification and the first description of a unique hemin/protoporphyrin-dependent transcriptional regulatory mechanism in mammalian cells. We have reported previously that the TRAP gene is transcriptionally upregulated by iron provided as diferric transferrin (FeTF), and we now report that, conversely, TRAP expres-
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The present results increase our understanding of the mTRAP promoter. The 5'-flanking region of mTRAP lacks a TATA box and contains an intron directly flanking the ATG. Although a putative iron response element has been identified in the 5'-flanking region of the porcine uteroferrin.
gene, no similar candidate iron response elements have been identified in the mTRAP 5′-flanking region.\textsuperscript{8,19} We have previously developed 5′ and 3′ deletion mutants of the mTRAP 5′-flanking region fused to a luciferase reporter gene to characterize regulation of TRAP expression.\textsuperscript{9} Transcriptional regulation of the mTRAP gene is complex. Two nonoverlapping regions of the 1.8-kb 5′-flanking region had promoter activity. An upstream promoter, P1, was located within the region from −881 bp to −463 bp relative to the ATG, whereas a downstream promoter, P2, was located in the intronic region between −363 bp and −1 bp. FcTF increased P1 promoter activity 2.5-fold and hemin decreased P1 promoter activity, but neither had any effect on P2 activity. Putative enhancers and suppressors for the P2 promoter were identified at −962 to −881 bp and at −1846 to −1240 bp, respectively. Based on those data and the data in this report, we have constructed a model for iron/hemin-responsive regulation of mTRAP expression (Fig 10).

To assess the generality of this type of regulation, we conducted a GenBank search for genes with significant homology to the 27-bp mTRAP candidate hemoresponse element. A large number of genes were identified, including 8 mammalian genes containing GAGGC tandem repeats in noncoding regions.\textsuperscript{20-23} Recently, Fleckenstein et al identified candidate tandem repeat GAGGC sequences in the 5′-flanking region of the hTRAP gene. These genes are listed in Table 1. They represent additional genes that may be under hemoresponse regulation. We also have evidence that the human protein kinase C β (PKCβ) gene is regulated in a similar fashion by iron\textsuperscript{24-25} and hemoresponse (unpublished observation). However, the iron/hemoglobin response area of the PKCβ gene is located in the 5′-untranslated region in an area for which nucleotide sequence data are not presently available. Therefore, it appears that there may be a family of mammalian genes that are regulated in similar fashion by iron and hemoresponse. However, additional studies will be required to determine whether these genes are, in fact, regulated by hemoresponse.

It is of interest that binding of HREBP to mTRAP 5′-flanking DNA was inhibited by anti-HAPI antibody (Fig 7). HAPI, a yeast protein that mediates heme activation of gene transcription, is a member of the GAL4 zinc finger class of transcription factors.\textsuperscript{26} Zhang and Guarente\textsuperscript{27} have defined the consensus DNA binding sequence for HAPI as a direct repeat of two CGG triplets with a 6-nucleotide spacer containing a critical TA sequence. Although the candidate mTRAP hemoresponse element contains two GGC triplets, HAPI does not bind to this inverted sequence,\textsuperscript{28} so that the two proteins may differ in their DNA binding requirements. An additional difference is that HAPI requires an interaction with heme to permit DNA binding,\textsuperscript{29} whereas HREBP appears to have a different mechanism of action, because prolonged incubation of cells with heme appears necessary for its interaction with oligonucleotide probes. Therefore, although differences from the yeast HAPI system clearly exist, our data suggest that hemoresponse regulation may be involved in expression of mammalian genes as well. However, additional studies are required to elucidate the relationship of HREBP to HAPI and to better understand the molecular mechanism by which it may interact with hemoresponse inhibition of transcription.

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Inhibition of tartrate-resistant acid phosphatase gene expression by hemin and protoporphyrin IX. Identification of a hemin-responsive inhibitor of transcription

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