Transcriptional Activation of the Heme Oxygenase Gene by Heme and Cadmium in Mouse Hepatoma Cells*

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Treatment of mouse hepatoma (Hepa) cells with heme or cadmium chloride in serum-free medium causes a rapid increase in the steady-state level of heme oxygenase (HO) messenger RNA. This increase is both dose- and time-dependent. Maximum accumulation of HO mRNA is observed 3 h after addition of either agent. Treatment of Hepa cells with heme or CdCl₂ also stimulates the transcription of the HO gene, as judged by in vitro nuclear transcription run-on assays. The maximum rate of HO gene transcription occurs 2 h after treatment with either agent. Comparison of the relative increase in the rate of HO gene transcription with the relative increase in the level of HO mRNA demonstrates that transcriptional activation is the primary mechanism by which heme and cadmium produce the accumulation of HO mRNA in Hepa cells. Cadmium may also influence other processes involved in the expression of HO, since the time course of mRNA accumulation diverges from that of gene transcription. However, neither heme nor cadmium alters the rate of HO mRNA degradation. Cobalt chloride and heat shock, which are potent inducers of HO mRNA in rat liver and rat C6 glioma cells, respectively, have only a small effect on the level of HO mRNA in mouse hepatoma cells.

Microsomal heme oxygenase catalyzes the rate-limiting step in heme catabolism, the oxidative degradation of heme to biliverdin (1). Under physiological conditions, the HO activity is highest in the spleen, where senescent erythrocytes are sequestered and destroyed (2). In contrast, the activity in the liver is approximately one-tenth of that observed in the spleen (1). However, HO activity is readily increased in liver and certain other tissues (2-4) by intravenous administration of heme(5) (in the form of hemin) and in cultured cells (5, 6) by addition of the substrate to the culture media. HO activity is also induced by a variety of substances including heme analogues (7), certain metal ions (8, 9), bromobenzene (10), endotoxin (11), and some hormones (6).

The mechanism by which the various agents induce HO activity is not completely understood. Incubation of cultured pig alveolar macrophages in the presence of exogenous heme increases the amounts of both HO protein and polysome-bound HO-specific mRNA in these cells (5). Similarly, translatable mRNA for HO is significantly increased in livers of rats treated with heme, cadmium chloride, or bromobenzene, suggesting that these agents act at the level of transcription to stimulate the synthesis of HO mRNA (4). Indeed, Shibahara et al. (12) recently demonstrated that heme treatment and heat shock effect the accumulation of HO mRNA in rat C6 glioma cells and that the heat shock response is regulated at the level of transcription. The present study examines the effect of heme and CdCl₂ on the level of HO mRNA in a highly differentiated hepatoma cell line, Hepa, originally derived from the mouse hepatoma BW 7756 (13). These cells are a good model system for investigating heme transport and catabolism (14).

The present study shows that both heme and CdCl₂ increase the steady-state level of HO mRNA in a dose- and time-dependent manner in Hepa cells. Furthermore, data from in vitro nuclear transcription assays and mRNA stability experiments suggest that heme- and cadmium-mediated accumulation of HO mRNA in Hepa cells results primarily from an increased rate of transcription of the HO gene.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases EcoRI and HindIII were purchased from Boehringer Mannheim, and Zeta-Probe nylon membrane was from Bio-Rad. Human placental ribonuclease inhibitor, vanadyl-ribonucleoside complex, and the nick translation DNA labeling kit were from Bethesda Research Laboratories. The random priming DNA labeling kit was from Pharmacia LKB Biotechnology Inc.; [α-32P]dCTP and [α-35S]UTP were from Du Pont-New England Nuclear. Mesoheme was from Forphyrin Products, and actinomycin D was from Sigma. All other chemicals were reagent grade.

Plasmids—The construction and isolation of plasmids pHRO1 (15), pA1 (16), and pL7 (17) containing cDNAs for rat heme oxygenase, chicken β-actin, and mouse ribosomal protein L7, respectively, have been reported. An internal 883-base pair EcoRI/HindIII restriction fragment from the rat HO cDNA was cloned into pUC 9 to generate plasmid pHRO1a.

Cell Culture and Treatments—Minimal deviation hepatoma cells from mouse solid tumor line BW 7756 were grown in Dulbecco's modified Eagle's medium supplemented with 0.38% glucose and 2% calf serum in a humidified atmosphere of 5% CO₂, 95% air at 37 °C. Routine subculturing was performed every 3-4 days. For RNA and nuclei isolation, subconfluent Hepa cells (1 - 3 x 10⁸ cells in 150-cm² culture flasks) were rinsed three times with 10 ml of prewarmed, serum-free Dulbecco's modified Eagle's medium buffered with 10 mm Heps, pH 7.2, and equilibrated in 5% CO₂ 95% air prior to use. The cells were subsequently incubated with 20 ml of the same medium containing the appropriate agent for the indicated time period. All incubations were at 37 °C unless indicated otherwise.

RNA and Nuclei Isolation and Northern Blot Analysis—Total
cellular RNA was isolated by the guanidinium isothiocyanate/cesium chloride method (18). Cytoplasmic RNA and nuclei were prepared from the same cell sample according to the procedure of Greenberg and Ziff (19). Nuclei were stored in liquid nitrogen for no more than 4 weeks prior to transcription assays.

RNA was fractionated on a denaturing formaldehyde-agarose (1%) gel, transferred to Zeta-Probe nylon membrane according to the manufacturer’s recommendation. Vacuum-baked filters were incubated for 4-16 h at 45 °C with 10 ml of hybridization solution: 3 × SSPE (1 × SSPE = 0.15 M NaCl, 1 mM EDTA, 10 mM sodium phosphate buffer, pH 7.4), containing 40% (v/v) deionized formamide, 5 mM sodium dodecyl sulfate (SDS),2 and 200 μg/ml denatured salmon sperm DNA. The filters were subsequently incubated with hybridization buffer containing the labeled DNA probe (specific activity: 2-10 × 10⁸ cpm/μl, 1-10 × 10⁸ cpm/ml solution) for 24-48 h at 45 °C. The HO probe was prepared by random priming (20) of the insert from plasmid pRH01a. β-actin and L7 probes were prepared by nick translation of plasmids pA1 and pL7, respectively. After hybridization, the filters were washed with solutions of 2× SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at room temperature for 30 min and 0.1% SSC, 0.1% SDS at 50 °C (for heterologous probes) or 65 °C (for homologous probes) for 60 min. The blots were then exposed to x-ray film (Kodak X-Omat AR) using an intensifying screen at -70 °C for 12-72 h.

In Vitro Nuclear Transcription Assays—In vitro nuclear transcription reactions were performed according to the method of Groudine et al. (21) with the modifications described by Rao et al. (22). Briefly, a 100-μl suspension of nuclei was mixed with an equal volume of reaction buffer to give the following final concentrations: 1-3 μM each of ATP, CTP, and GTP, 50 units of rabbit reticulocyte RNA polymerase (Boehringer-Mannheim) in 1 ml of hybridization buffer containing the labeled DNA probe (specific activity: 2-10 × 10⁸ cpm/μl, 1-10 × 10⁸ cpm/ml solution) for 72 h at 30 °C. The reaction was terminated by the addition of 600 μl of a DNase I (40 μg/ml) solution. After 15 min at 30 °C, SDS, EDTA, and RNA were added to give final concentrations of 1%, 2.5 mM, and 200 μg/ml, respectively. The reaction mixture was further incubated at 37 °C for 30 min and then extracted twice with a 1:1 mixture of phenol/chloroform in the presence of 100 μg/ml tRNA. The DNA-bound filters were initially incubated in hybridization solution (the same as that employed in Northern blot analysis, except that Tris was substituted for salmon sperm DNA) and then incubated with labeled RNA in 1 ml of hybridization solution for 72 h at 45 °C. Washing conditions were equivalent to those employed for Northern blot analysis. Filters were exposed to x-ray film (Kodak X-Omat AR) for 18-72 h. Incubation of spent hybridization medium with a second filter containing bound plasmid showed that >90% of the particular RNA had hybridized to the initial filter.

RESULTS

Treatment of mouse Hepa cells with varying concentrations of heme or CdCl₂ in serum-free medium for 4 h increases the steady-state level of HO mRNA in a dose-dependent manner (Fig. 1). Ten micromolar heme consistently increases the amount of HO mRNA from 20- to 25-fold over that of control levels, as measured by liquid scintillation counting of appropriately excised areas of the RNA blot filters (data not shown). A similar increase (25-30-fold) in the level of HO mRNA is observed with 10 μM CdCl₂, although incubation with 20 μM CdCl₂ produces the maximal accumulation of HO mRNA (~40-fold).

The time course of HO mRNA accumulation during treat-

3 The abbreviations used are: SDS, sodium dodecyl sulfate; MT, metallothionein.

![FIG. 1. Dose-dependent increase in the level of heme oxygenase mRNA in Hepa cells treated with heme or cadmium. Hepa cells (~2 × 10⁶ cells/flask) were incubated for 4 h in 20 ml of serum-free medium containing 0, 0.2, 0.5, 1.0, 2.0, 5.0, 7.5, and 10 μM heme (A, lanes a–h, respectively) or 0, 2.5, 5.0, 10, 20, 30, 40, and 50 μM CdCl₂ (B, lanes a–h, respectively). Total RNA was isolated, and 20-μg samples were electrophoresed and transferred to Zeta-Probe membrane. Northern blot analysis was carried out as described under “Experimental Procedures” using the insert of plasmid pRH01a as the hybridization probe. The filter was autoradiographed for 12 h. The migration of the 28S and 18S ribosomal RNA species is indicated.](image1.png)

![FIG. 2. Time course of the accumulation of heme oxygenase mRNA in Hepa cells treated with heme or cadmium. Hepa cells (~2 × 10⁶ cells/flask) were incubated in 20 ml of serum-free medium containing 10 μM heme (A) or 10 μM CdCl₂ (B) for 0, 1, 2, 3, 4, 5, and 6 h (lanes a–g, respectively) or in 20 ml of medium containing control solvent for 6 h (lane h). Total RNA was isolated, and 20-μg samples were electrophoresed and transferred to nylon membranes. Northern blot analysis was carried out as described under “Experimental Procedures.” The filter was exposed to film for 12 h. The HO probe was melted, and the filter was hybridized to a mixture of nick-translated plasmids pA1 (β-actin) and pL7 (mouse L7 ribosomal protein). The filter was autoradiographed for 12 h. The migration of the 28S and 18S ribosomal RNA species is indicated.](image2.png)
and ribosomal protein L7 mRNA do not change appreciably during the time course of these treatments. Intravenous injection of cobalt chloride into rats significantly induces HO mRNA in the liver (15), and incubation at 42°C of rat C6 glioma cells causes at least a 20-fold increase in HO mRNA (12). In contrast, treatment of mouse Hepa cells with 50 μM CoCl\(_2\) or incubation of Hepa cells at 42°C produces only a small, time-dependent increase (≈2-3-fold) in the level of HO mRNA over a 6-h period (Fig. 3). This increase is significantly less than that (20-40-fold) observed in heme- or CdCl\(_2\)-treated Hepa cells.

To establish the mechanism(s) by which heme and CdCl\(_2\) increase the steady-state level of HO mRNA in Hepa cells, *in vitro* transcription assays were performed with nuclei isolated from cells treated with heme or CdCl\(_2\). In initial experiments, Hepa cells were treated with either 10 μM heme or 10 μM CdCl\(_2\) in serum-free medium prior to nuclei isolation. However, part of the nuclei pellet from heme-treated cells was difficult to resuspend, probably due to structural damage to the heme-containing nuclei during the isolation process. This problem, which was not encountered in cadmium-treated cells, was alleviated by reducing the concentration of heme in the medium to 5 μM. Preliminary studies indicated that the initial rate of UMP incorporation and the level of RNA polymerase II activity (60% of total transcription) were similar in nuclei isolated from control and treated cells. Furthermore, in any given experiment, the amount of trichloroacetic acid-precipitable counts/nuclei was similar between nuclei from control and treated cells. As shown in Fig. 4, both agents significantly increase the rate of transcription of the HO gene in isolated nuclei. This induction is very rapid, with the maximal transcription rate occurring within 2 h of treatment. The rate of HO gene transcription in nuclei from heme-treated cells then declines rapidly and approaches that of control levels by 5-6 h. In contrast, the rate of HO gene transcription in nuclei from cadmium-treated cells declines gradually, remaining significantly higher than control levels even after 6 h. The rate of *in vitro* transcription of the genes for β-actin and L7 ribosomal protein is not affected by treatment of Hepa cells with heme or CdCl\(_2\).

To compare the steady-state level of HO mRNA with the rate of HO gene transcription in Hepa cells, cytoplasmic RNA and nuclei were isolated from the same cell sample. The time course of HO mRNA accumulation and of *in vitro* HO gene transcription (relative to control levels) in cells treated with heme or CdCl\(_2\) are presented in Fig. 5. During both treatments, the rate of transcription precedes the maximal accumulation of HO mRNA by approximately 1 h, and the maximal increase, relative to control levels, in the rate of HO gene transcription and in the level of cytoplasmic HO mRNA appear quantitatively equivalent. However, there seem to be differences in the response to heme and cadmium. While the HO mRNA levels appear to follow closely in response to the changes in transcription in heme-treated cells, maximal levels of HO mRNA are sustained even after the rate of transcription declines in cadmium-treated cells. This prolonged accumulation of HO mRNA suggests that cadmium may alter cellular processes other than transcription.

One such process could be the degradation of HO mRNA. Consequently, the effect of heme and CdCl\(_2\) on the rate of degradation of HO mRNA was examined by measuring the steady-state level of HO mRNA as a function of time in the presence of the transcription inhibitor actinomycin D. Preliminary experiments indicated that 5 μg/ml actinomycin D inhibited RNA synthesis by 95% in Hepa cells. The half-life (t\(_{1/2}\)) of cadmium-induced HO mRNA, 5.3 h, is only slightly altered in the presence of heme (t\(_{1/2}\) = 5.7 h) or CdCl\(_2\) (t\(_{1/2}\) = 6.2 h) (Fig. 6). Under conditions of constant synthesis, increasing the half-life of a mRNA species from 5.3 to 6.2 h increases the steady-state level of that mRNA by only 17% (23). Consequently, the change in the rate of HO mRNA degradation cannot completely account for the difference between the level of HO mRNA and the rate of *in vitro* transcription during exposure to cadmium (see Fig. 5). The half-life of uninduced HO mRNA, approximately 5 h, is not appreciably affected by heme, and the rate of degradation of heme-induced HO mRNA (t\(_{1/2}\) = 5.5 h) is also not affected by either agent (data not shown).

**DISCUSSION**

Exposure to heme stimulates heme oxygenase activity in a variety of tissues *in vivo* and in cultured cells. Polysome-
Fig. 5. Kinetics of heme oxygenase mRNA accumulation and heme oxygenase gene transcription in Hepa cells treated with heme or cadmium. Hepa cells (~2 x 10^6 cells/flask) were incubated in 20 ml of serum-free medium containing 5 μM heme (A) or 10 μM CdCl₂ (B), and, at the indicated time, cytoplasmic RNA and nuclei were isolated. Ten-microgram samples of RNA were blotted onto Zeta-Probe membrane, and the filters were incubated with labeled HO probe or β-actin probe. In vitro transcription reactions were carried out as described under “Experimental Procedures.” Labeled transcripts (2 x 10^6 cpm/time point) were hybridized to membranes containing bound plasmid DNA for 72 h. Filter-bound radioactivity was quantitated by liquid scintillation counting. Relative HO mRNA levels (0-0) and gene transcription rates (C-D) were calculated by subtracting background radioactivity, normalizing these values to the level of β-actin mRNA or gene transcription at each time point, and dividing by the control (time = 0 h) value. These ratios are plotted as a function of time. Each data point represents the average value from two individual experiments.

Fig. 6. The effect of heme and cadmium on the rate of degradation of heme oxygenase mRNA in Hepa cells. Hepa cells (~1 x 10^6 cells/flask) were incubated in 20 ml of serum-free medium containing 10 μM CdCl₂ for 3 h. This medium was removed, and the cells were incubated in 20 ml of medium containing control solution (A), 10 μM heme (B), or 10 μM CdCl₂ (C) for the indicated time. Total RNA was isolated, and 5-μg samples were blotted onto Zeta-Probe membrane. The filters were hybridized with the labeled insert from plasmid pHO1a, and the bound radioactivity was quantitated by liquid scintillation counting. Each data point represents the mean value from three separate experiments. The standard deviation is indicated by the error bars, and each line represents the best fit from a linear regression analysis of the data points. The half-lives calculated from the graphs are 5.3 h (A), 5.7 h (B), and 8.2 h (C).

The level of stimulation by heme are equivalent to those observed in rat C6 glioma cells. Furthermore, the present investigation directly demonstrates that heme increases the rate of HO gene transcription and that this stimulation of transcriptional activity is the primary mechanism by which heme affects the accumulation of HO mRNA. This conclusion is based on two separate observations: 1) the increase in the level of HO mRNA (relative to control values) is quantitatively equivalent to the increase in the rate of HO gene transcription in Hepa cells treated with heme and 2) the rate of degradation of HO mRNA is only slightly retarded by heme.

While heme is thought to regulate the expression of certain proteins such as 5-aminolevulinate synthase (24) and cytochrome P-450 (25) at the level of transcription, little is known about the mechanism by which heme modulates gene transcription in mammalian cells. In yeast, heme acts as a positive effector to induce the synthesis of at least three components of the respiratory chain: iso-1-cytochrome c, iso-2-cytochrome c, and cytochrome-b₅ (26-28). Heme mediates this response via transcriptional activation of the genes encoding these proteins. This activation requires the binding of at least three different proteins to upstream activation sites on the genes, and the binding of one of these factors, HAP1, is significantly enhanced by the presence of heme (29). The sites on the iso-1-cytochrome c and iso-2-cytochrome c genes with which HAP1 interacts have been determined (29, 30). The rat HO gene (see below) contains no sequence motifs similar to these elements.

The rat heme oxygenase gene was recently isolated and sequenced (31). Composed of 6830 nucleotides, the gene is organized into four introns and five exons. The 5′-flanking region contains sequences similar to previously identified DNA elements considered to be binding sites for various transcriptional factors. Among the sequences is a motif that contains two copies of core sequences of the metal regulatory elements found in metallothionein (MT) genes (32). The presence of such a sequence in the rat HO gene suggests that heavy metal stimulation of HO activity is regulated at the

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level of transcription in a manner analogous to that demonstrated for MT (reviewed in Ref. 33). Consistent with this prediction, cadmium is shown here to significantly increase the steady-state level of HO mRNA and the rate of transcription of the HO gene in mouse hepatoma cells. Furthermore, both the time course and extent of HO mRNA accumulation and HO transcription are qualitatively and quantitatively equivalent to those of MT mRNA accumulation and MT transcription in cadmium-treated Hepa cells (data not shown).

In contrast to the effects of cadmium chloride, cobalt chloride causes only a small alteration in the level of HO mRNA in Hepa cells. This *in vitro* result differs from the dramatic increases in hepatic HO activity (8, 9) and hepatic HO mRNA (15) in CoCl2-treated rats. However, Durnam and Palmer (34) observed that 300 μM CoCl2 was necessary for optimal induction (~9-fold) of MT1 mRNA in a subclone of the parental Hepa cell line and that 40 μM CoCl2 increased the amount of MT1 mRNA only 2–3-fold over control levels. These data are consistent with our observation on the effect of 50 μM CoCl2 on the level of HO mRNA in Hepa cells. The similarities between HO and MT mRNA induction described above suggests that similar, if not identical, mechanisms are involved in the regulation of HO and MT expression by heavy metals. While the mouse HO gene has not been sequenced, it probably contains one or more sequence motifs related to the MRE found in metallothionein genes.

In mouse Hepa cells, the cadmium-induced accumulation of HO mRNA results primarily from transcriptional activation of the HO gene. However, the observation that the time course of HO mRNA accumulation does not completely parallel the time course of HO *in vitro* transcription suggests that cadmium may alter other processes involved in the normal expression of HO. Among these processes are (see Raghow (35) for recent review): 1) synthesis and/or mobilization of trans-acting factors involved in HO mRNA accumulation, 2) processing of the primary transcript, 3) modification of the 5' and 3' termini, 4) transport of mRNA into the cytoplasm, and 5) degradation of mRNA. Our results indicate that cadmium produces a slight but measurable increase in the half-life of HO mRNA in Hepa cells. However, this alteration in the rate of degradation cannot completely account for the difference in the kinetics of HO mRNA accumulation and of *in vitro* HO gene transcription.

The 5'-flanking region of the rat HO gene also contains a sequence motif that is identical to the consensus sequence of the heat shock element (reviewed in Ref. 36), providing an explanation for the more than 20-fold increase of HO transcripts in rat C6 glioma cells incubated at 42 °C (12). In contrast, human HO is not responsive to heat shock (37). Our observations suggest that mouse HO is apparently not a heat shock protein, since only a moderate 2–3-fold induction of HO mRNA was observed in Hepa cells. However, *in vivo* studies with mice are necessary to substantiate this conclusion. The reason for this species-based difference is not clear, but quite possibly neither the mouse nor the human HO genes contain the cis-acting heat shock element. Currently, no information is available on the structure of these HO genes.

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REFERENCES