Induction of Several Acute-Phase Protein Genes by Heavy Metals: A New Class of Metal-Responsive Genes†

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ABSTRACT: Acute-phase reactants, metallothioneins, and heat-shock proteins are the products of three families of genes that respond to glucocorticoids and cytokines. Metallothioneins and heat-shock proteins, however, are also stimulated by heavy metals, whereas very little is known about the effect of heavy metals on acute-phase-reactant genes. We have studied the effect of heavy metals (Hg, Cd, Pb, Cu, Ni, and Zn) and Mg on the acute-phase reactants α_1-acid glycoprotein, C-reactive protein, α_1-antitrypsin and α_1-antichymotrypsin. α_1-Acid glycoprotein and C-reactive protein mRNA levels were increased severalfold in livers of heavy-metal-treated Balb/c mice. The strongest induction was mediated by Hg, followed in order of response by Cd > Pb > Cu > Ni > Zn > Mg. None of the metals affected the mRNA levels of albumin, α_1-antitrypsin, and α_1-antichymotrypsin. Furthermore, failure to repress albumin, a negative acute-phase reactant, indicated that the induction of these genes was not due to a metal-mediated inflammatory response. The metals also induced α_1-acid glycoprotein and C-reactive protein in adrenalectomized animals, indicating that induction by the heavy metals is not mediated by the glucocorticoid induction pathway. Sequence analysis has revealed a region of homology to metal-responsive elements in the α_1-acid glycoprotein and C-reactive protein promoters. Additionally, an α_1-acid glycoprotein expression vector, pAGP(−595)CAT, responded to Hg and Cd when transfected into human HepG2 cells. Our studies indicate that the induction of α_1-acid glycoprotein and C-reactive protein by heavy metals may be regulated by these metal-responsive elements at the level of transcription.

Eukaryotic organisms possess natural defense processes triggered by stress factors such as injury, infection, and inflammation, which are the processes by which cells and tissues are protected against further damage and injury and by which restorative processes are initiated (Kushner, 1982). The acute-phase reactants (APR), metallothioneins (MT), and heat-shock (HS) proteins are the products of three families of genes that are induced by various physical and chemical stress stimuli (Koj, 1974; Oh, et al., 1978; Nover, 1984). The APR and MT families of genes, for example, respond to inflammation caused by bacterial lipopolysaccharide (LPS) or turpentine and to mediators of the inflammatory response such as glucocorticoids and cytokines (Sobocinski et al., 1981; Sobocinski & Canterbury, 1982; Duram et al., 1984; Hamer, 1986; Baumann, 1989). Metallothioneins and HS proteins, however, are also stimulated by heavy metals such as Cd, Pb, Hg, and Zn (Nover, 1984; Hamer, 1986). The response of MT genes to these metals is mediated by the interaction of trans-acting factors with cis-acting DNA sequences or metal-responsive elements (MRE) in their promoter regions (Mayo et al., 1982; Carter et al., 1984; Karin et al., 1984; Searle et al., 1985; Imbert et al., 1989). However, there is very little information on the effect of heavy metals on the expression of APR genes in general or on that of specific members of this family of genes. Harrison et al. (1986) reported that methylmercury induces an increase in serum α_1-acid glycoprotein (AGP) levels in the rat, and Argawal and Bhattacharya (1989) have shown that serum levels of C-reactive protein (CRP) are increased in Hg-treated rats. These investigators also showed that Hg is strongly bound to the sulfhydryl groups of purified *Limulus* CRP. It has also been reported that Pb induces increased synthesis in the liver of a group of acid serum proteins that, although not identified, may belong to the APR family (Nicholls et al., 1984). Sequence analysis of the mouse AGP-1 gene revealed the presence of four MRE consensus sequences, three of which are located in the 5′-flanking region and one of which is located in intron 5 (Cooper et al., 1987). Furthermore, these MREs overlap the glucocorticoid receptor element (GRE) in the proximal promoter of the AGP-1 gene at −108 to −127 and −135 to −149. The presence of MREs in the promoter region and within the mouse AGP-1 gene suggested to us that this gene, and possibly the other genes of this locus, i.e., AGP-2 and AGP-3, may be inducible by heavy metals.

The studies cited above indicate that several APRs, i.e., AGP, CRP, and the unidentified acid serum proteins, may be inducible by heavy metals. On the basis of the similarity of factors that regulate AGP, MT, and HS protein genes as well as the presence of consensus MRE sequences in the promoter of the AGP-1 gene, we conducted experiments to determine if heavy metals such as Hg, Cd, Pb, Cu, Ni, and Zn can induce the expression of various members of the APR family of genes and to determine whether the MREs in the promoter of the AGP-1 gene are functional.

Experimental Procedures

Animals. Normal and adrenalectomized male Balb/c mice (19–20 g) were purchased from Charles River Breeding Laboratory. All mice were maintained under 12-h light–dark cycle and provided free access to food and water or 0.9% NaCl

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for the adrenalectomized mice. $HgCl_2$, $CdCl_2$, $ZnCl_2$, $CuCl_2$, $PbCl_2$, $NiCl_2$, and $MgCl_2$ or LPS were injected ip in 0.1 mL of pyrogen-free saline. Dexamethasone was first dissolved in ethanol (1 mg/mL) and then further diluted in saline. In each experiment, all mice were injected at the same time (9:00–10:00 a.m.) and tissues were removed at the indicated times following each treatment. Peritoneal macrophages were isolated as described previously (Rokita et al., 1987; Yiangou & Hadjipetrou-Kourounakis, 1989). After the final wash, the cells were pelleted and RNA was isolated immediately by use of the RNAzol B kit.

Isolation and Northern Analysis of RNA. Total RNA from liver, kidney, spleen, or brain was isolated by the method of Chirgwin et al. (1979). Tissues were homogenized in 7.5 M guanidine hydrochloride/1% 2-mercaptoethanol/0.1% lauroSarcosine/25 mM sodium citrate. The homogenate was layered over a 5.7 M CsCl cushion, and RNA was separated by centrifugation. Total RNA from macrophages was isolated by use of the RNAzol B method as recommended by the manufacturer (CINNA/BIOTECX Laboratories International Inc., Friendswood, TX 77546) and on the basis of the method previously described by Chomczynski et al. (1987). Purified RNA (10 μg/sample) was resolved by electrophoresis through a formaldehyde denaturing agarose gel (1.4%) buffered in 0.02 M morpholinopropanesulfonic acid/1 mM EDTA, pH 7.4. Equal RNA loading was tested by comparison of rRNA intensities in ethidium bromide stained gels. The RNA was then transferred to nitrocellulose membranes as described by Maniatis et al. (1982) and baked for 2 h at 80 °C under vacuum. AGP, AT, ACh, and albumin mRNAs were detected by hybridization with ^{32}P-labeled RNA probes prepared in an in vitro transcription reaction in the presence of [α-^{32}P]UTP (3000 Ci/mmol; ICN Radiochemicals, Irvine, CA 92713). Mouse metallothionein II or transferrin mRNAs were detected by hybridization with ^{32}P-labeled cDNA prepared by nick translation using [α-^{32}P]dCTP (3000 Ci/mmol; ICN Radiochemicals). The filters were prehybridized for 4 h at 65 °C in a solution containing 4× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 50% formamide, 0.2 mg/mL sheared and denatured salmon sperm DNA, 4× Denhart's solution, 0.1% SDS, and 50 mM $NaPO_4$ buffer, pH 6.5 (Maniatis et al., 1982). Hybridization was done in fresh solution containing (2–5) × 10^6 cpm/mL of probe for 12 to 16 h. Filters were washed four times for 20 min at 22 °C in 2× SSC/0.1% SDS and four times for 30 min at 65 °C in 0.1× SSC/0.1% SDS and then air-dried and exposed to Kodak XAR-5 film. Quantitation of the data was done by densitometric scans of autoradiographs. To detect CRP mRNA, filters were hybridized to a specific oligonucleotide probe representing the sequence from 495 to 518 (Ohnishi et al., 1988). The oligonucleotide probe was labeled at the 5′ end as described by Dente et al. (1987) using T4 polynucleotide kinase in the presence of [^{32}P]ATP (3000 Ci/mmol; ICN Radiochemicals). Theoretical hybridization temperatures were calculated from the formula T_m = (2 °C)(A + T) + (4 °C)(G + C) (Wallace & Miyada, 1987) and hybridization was done 12 °C below the T_m. Prehybridization was done for 4 h in a solution containing 1 M NaCl, 4× Denhart's solution, and 0.1 mg/mL salmon sperm DNA. Hybridization was done under optimum hybridization temperatures in fresh solution at 55 °C for the CRP probe (3 × 10^6 cpm/mL) for 17 to 20 h, and then the filters were washed three times for 15 min at 22 °C in 6× SSC/0.1% SDS and treated as described above.

Plasmid Constructs. A *Bam*HI/*Ban*I 613-bp fragment of the mouse AGP-1 gene extending from −595 to +18 was isolated from a Charon 28 λ genomic clone (Cooper et al., 1987), blunt-ended by treatment with Klenow fragment and deoxynucleoside triphosphates, and ligated into the *Hin*cII sites of pUC18. The plasmid pAGP(−595)CAT was constructed by removing the fragment from pUC18 by digestion with *Bam*HI and *Hin*dIII and inserting it into *Bam*HI/*Hin*dIII-cut pP5-CAT vector (Gorman et al., 1982; Widen & Papaconstantinou, 1986). In this vector derived from pSV2-CAT (Gorman et al., 1982), the *Bam*HI site 3′ to the CAT gene has been deleted and another *Bam*HI site was created 5′ to the *Hin*dIII site of pSV2-CAT (Widen & Papaconstantinou, 1986).

Tissue Culture, Cell Transfections, and Analyses. HepG2 cells were purchased from American Type Culture Collection (ATCC), maintained in modified Eagle's medium plus 10% bovine calf serum, and passaged at confluence. Cells (1 × 10^6) were plated in 100-mm petri dishes and 24 h later were transfected with 3 pmol of plasmid DNA by calcium phosphate precipitation (Graham & Van Der Eb, 1973); the pCH110 plasmid (0.75 pmol) was also included in the precipitation mix. The cells were allowed to recover from the transfection process for 36 h, and fresh medium containing heavy metal salts, dexamethasone, or both at the indicated concentrations was added. After 24 h, cells were harvested by scraping and lysed by freeze–thawing in 200 μL of 0.25 M Tris-HCl (pH7.8)/0.005 M dithiothreitol. Protein concentration was determined by the method of Bradford (1976), and 120 μg of protein was used for determination of β-galactosidase activity (Miller, 1972). CAT enzyme activity was determined as described previously (Gorman et al., 1982). Prior to performing the CAT assay, cell extracts were heated at 60 °C for 5 min to inactivate endogenous deacetylase activity (Prowse & Baumann, 1988). The relative efficiency of transcription of the CAT gene was expressed as a ratio of the total amount of acetylated [^{14}C]chloramphenicol per microgram of protein per hour and normalized to β-galactosidase activity (Izban & Papaconstantinou, 1989).

Results

Induction of AGP Gene Expression by $HgCl_2$. To initiate our investigation on the induction of APR by heavy metals, adult male Balb/c mice (19–20 g) were injected intraperitoneally (ip) with different doses of $HgCl_2$, and 18 hours postinjection total hepatic RNA was analyzed for changes in the level of the AGP mRNA pools (Figure 1). Doses between 0.5 and 1.75 mg/kg of body weight induced approximately the same increase in AGP mRNA levels (Figure 1B). Elevated hepatic AGP mRNA levels after a single ip injection of 0.5 mg of $HgCl_2$/kg of body weight were detected 3 h postinjection and were maximal 6–24 h postinjection (Figure 1A). By 3 h, the AGP mRNA levels increased 3–5-fold, and an increase of 9–12-fold was observed by 6–24 h. Return to control levels occurred at approximately 48 h postinjection (Figures 1D and 5A). Processing of newly synthesized AGP mRNA results in the transient appearance of a relatively stable mRNA intermediate (~1.1 kb) that is larger than the more stable mature mRNA (~0.9 kb) detected in control samples (Figure 1D). This difference in size is due to deadenylation of the mRNA intermediate (Shiels et al., 1987; Carter et al., 1989a). The higher molecular weight AGP mRNA clearly seen in the 3- and 6-h samples was progessively reduced in size, indicating that AGP mRNA induction and processing in Hg-treated animals is similar to that described during experimentally induced inflammation in rats (Shiels et al., 1989) and during glucocorticoid or monokine stimulation of HTC cells or rat primary hepatocytes (Carter et al., 1989a,b).

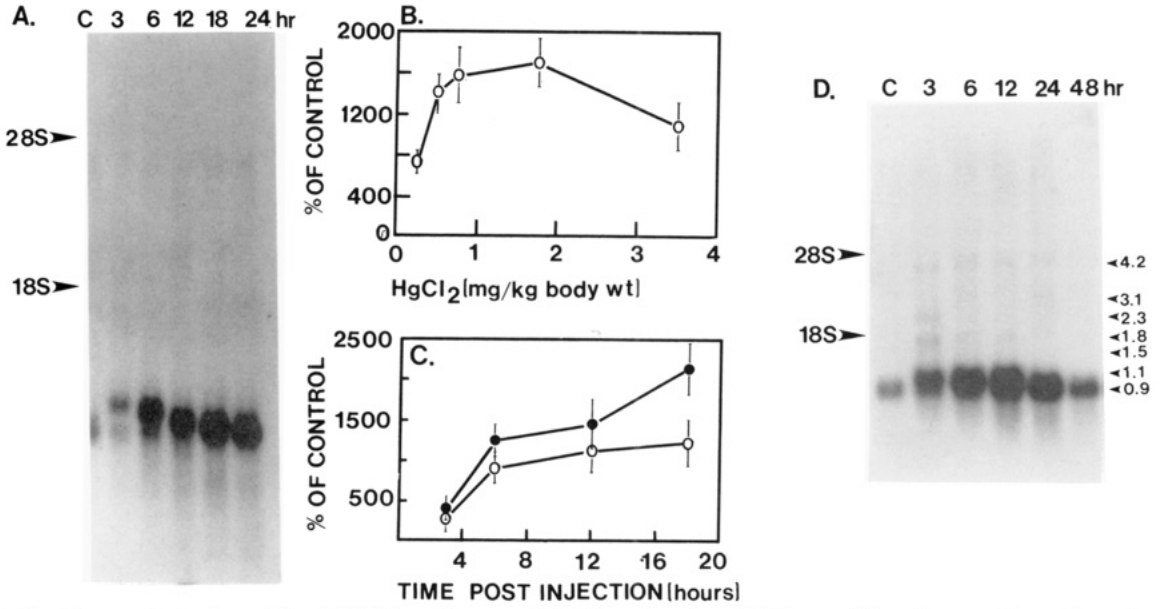

FIGURE 1: Northern analysis of total liver RNA from Hg-treated Balb/c male mice. $HgCl_2$ was injected intraperitoneally, and total RNA was extracted at the indicated time intervals. Total RNA (10 μg/sample) was separated by agarose gel electrophoresis and analyzed by Northern blot hybridization with a specific AGP RNA probe derived from the in vitro transcription of a mouse AGP cDNA plasmid, pT3/T7 MAGP4. (A) The autoradiogram shows the appearance, accumulation, and subsequent deadenylation of the high molecular weight AGP mRNA in the liver of mice treated with $HgCl_2$. (B) The dose–response curve shows the accumulation of AGP mRNA in the livers, 18 h postinjection of $HgCl_2$. (C) The curve shows the comparison of AGP mRNA accumulation in the livers of mice treated with LPS (●) and $HgCl_2$ (○). Autoradiographic bands were scanned and expressed as a percentage of the control samples. Each point represents an average of three experiments. (D) The autoradiogram shows the major intermediates of RNA processing that appear in the early phases of induction by Hg. The sizes of the intermediates are greater than that of the AGP mRNA and were estimated by use of the 18S and 28S RNAs as size references. The numbers on the right side of the autoradiogram correspond to the sizes of the intermediates in kilobases.

Potential intermediates of mRNA processing ranging in size from 4.2 to 1.5 kb were detected at 3 h postinjection and then gradually decreased and completely disappeared by 48 h (Figure 1D). The same size intermediates were observed in liver nuclear RNA samples from LPS-treated mice. The above results suggest that these RNAs are probably intermediates of processing of AGP mRNA primary transcripts and that treatment with $HgCl_2$ induces the transcription of the mouse AGP gene.

AGP mRNA was induced by both Hg and LPS and reached near maximal levels by 6 h after treatment with heavy metal (Figure 1C). However, after 6 h, AGP mRNA levels continued to increase in LPS-treated animals while Hg-mediated AGP mRNA levels remained constant. In general, it has been our experience that doses of LPS ranging from 0.5 to 1.0 mg/kg of body weight induce significantly higher AGP mRNA levels than $HgCl_2$. Although we do not know the basis for the continued increase in the mRNA pool in LPS-treated livers, these studies do point to possible differences between the two processes of induction. Metal administration did not induce extrahepatic AGP mRNA production in kidney, spleen, or brain, indicating that the response to heavy metals may be liver-specific (data not shown).

Effect of $HgCl_2$ on Albumin Production. During the acute-phase response, transcription of the albumin gene is repressed and albumin mRNA levels are decreased significantly (Kulkarni et al., 1985; Darlington et al., 1986). Albumin belongs, therefore, to the group of proteins that are negatively regulated during the acute-phase response. In our experiments, albumin mRNA levels in Hg-treated mice were the same as in the control animals (Figure 2A). However, the albumin mRNA levels in the livers of mice injected with LPS (0.5 mg/kg of body weight) were 40–50% lower than those observed in control or Hg-treated mice (Figure 2B). The above results suggest that Hg does not induce a general acute-phase response during which albumin transcription would be down-regulated and that the Hg-mediated induction of AGP gene expression is probably independent of the pathway of the acute-phase response.

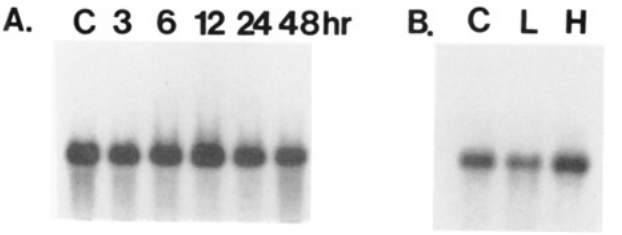

FIGURE 2: Northern analysis of the albumin mRNA levels in the livers of $HgCl_2$- or LPS-treated mice. (A) Albumin mRNA levels are shown in Hg-treated animals from 3 to 48 h postinjections. (B) Albumin mRNA levels are shown in livers of control (C), LPS (L), and $HgCl_2$ (H) treated mice 15 h postinjection. RNA was hybridized by use of a specific albumin RNA probe derived from the in vitro transcription of a mouse albumin cDNA plasmid, pT7/T3ALB, cDNA.

Induction of AGP Gene Expression in Adrenalectomized Mice by $HgCl_2$. Glucocorticoids are involved in the regulation of the AGP gene and are believed to be required for its maximum expression in vivo and in vitro (Baumann et al., 1983a,b, 1987; Reinke & Feigelson, 1985; Baumann & Maquat, 1986; Klein et al., 1987; Marinkovic et al., 1989). To examine whether Hg-mediated induction of the AGP gene was dependent upon glucocorticoids, adrenalectomized mice were injected with $HgCl_2$ (0.5 mg/kg of body weight) and their total liver RNA was analyzed to compare the pool levels of specific AGP and albumin mRNAs. The results presented in Figure 3 show that the increase in AGP mRNA pool was similar in the adrenalectomized and nonadrenalectomized mice (Figure 1A). To further establish that the Hg-mediated induction of AGP mRNA is independent of glucocorticoid interactions, adrenalectomized mice were treated ip with dexamethasone (0.5 mg/kg of body weight) and 1 h later received $HgCl_2$; control animals received either dexamethasone or $HgCl_2$. As

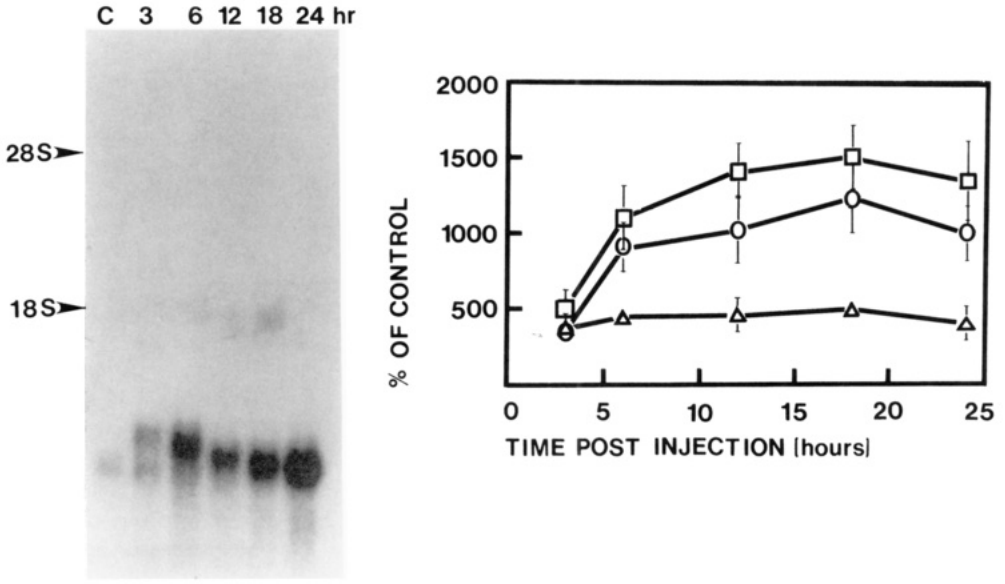

FIGURE 3: $HgCl_2$-mediated induction of AGP mRNA levels in the livers of adrenalectomized mice. Adrenalectomized mice were used at least 1 week after removal of the adrenal glands. (A) Accumulation and subsequent deadenylation of the high molecular weight AGP mRNA in the liver of $HgCl_2$ treated adrenalectomized mice is shown. (B) The curves show the effect of dexamethasone and $HgCl_2$ on the induction of AGP mRNA levels in adrenalectomized mice: $HgCl_2$ (○); dexamethasone (△); dexamethasone + $HgCl_2$ (□). Adrenalectomized mice were injected ip with dexamethasone, and 1 h later half of the animals received an injection of $HgCl_2$. Each time point represents an average of three experiments.

expected, the Northern analysis showed that dexamethasone does induce AGPmRNA. However, the dexamethasone treatment did not have a synergistic effect on the Hg-mediated induction of the AGP gene (Figure 3). Furthermore, since the error bars in the Hg and Hg + dexamethasone response data superimpose at all but the 12-h point, we interpret these data to indicate that the response after Hg + dexamethasone treatment is not additive. These results support our proposal that glucocorticoid interactions are not involved in the Hg-mediated regulation of the AGP gene.

Effect of $HgCl_2$ on Other APR Genes in Normal and Adrenalectomized Mice. The mRNA levels of three other APRs, α_1-antitrypsin (AT), α_1-antichymotrypsin (ACh), and C-reactive protein (CRP), were investigated to determine whether the expression of these genes is affected by $HgCl_2$.

To detect specific CRP mRNA pools, we used an oligonucleotide whose sequence is in the second exon of the mouse CRP gene from position 494 to position 518 relative to the transcription initiation site (Ohnishi et al., 1988). In normal Hg-treated mice, elevated hepatic CRP mRNA levels were detected by 3 h postinjection, were maximal by 6 h (5–7-fold higher than the control), and were reduced to constitutive levels by 18–24 h (Figure 4C). However, a smaller increase of the CRP mRNA levels was observed in total liver RNA from Hg-treated adrenalectomized mice, suggesting that glucocorticoids may be required for the maximal response of the CRP gene to Hg treatment (Figure 4C). During the inflammatory response induced in mice by LPS, hepatic CRP mRNA levels were 2–3 times higher than the Hg-mediated mRNA levels (Figure 4D). Furthermore, the CRP mRNA levels in the LPS-treated mice appear to reach maximal levels by 3 h postinjection, while the mRNA levels in the Hg-treated mice were maximal by 6 h postinjection. These results suggest that LPS is a stronger inducer of CRP than of $HgCl_2$ and that glucocorticoids may be required for an efficient Hg-mediated CRP gene induction.

Levels of AT mRNA were not affected by Hg treatment in livers of both normal (Figure 4A) and adrenalectomized mice (data not shown), and no increase was observed in LPS-treated mice. However, analysis of mRNA levels in peritoneal macrophages of LPS-treated mice revealed an increase in AT mRNA levels, whereas no increase was observed in macrophages of Hg-treated animals (Figure 4B). Finally, our analyses showed that the level of ACh mRNA, which is very low in control animals, was not affected by Hg treatment. These data indicate that the ability of Hg to induce expression of APR genes is thus far limited to AGP and CRP.

Effect of Other Heavy Metals on the Induction of AGP and CRP mRNAs. The heavy metals Cd, Zn, Cu, Hg, Pb, and Ni induce metallothionein gene expression in the livers of mice (Durnam & Palmiter, 1981; Hamer 1986). To investigate the effect of heavy metals on the acute-phase response, mice were injected with $HgCl_2$, $CdCl_2$, $ZnCl_2$, $PbCl_2$, $CuCl_2$, and $NiCl_2$ (0.5 mg/kg of body weight). Total liver RNA was isolated at various times from 3 to 48 h postinjection and subjected to Northern analysis to compare specific AGP, AT, ACh, CRP, metallothionein II (MT II), and albumin mRNA pools. The data in Figure 5A,B show that AGP mRNA levels of metal-treated mice were significantly increased. The strongest response was produced by Hg and is followed by Cd > Pb > Cu > Ni > Zn (Figure 4A,B). Surprisingly, $MgCl_2$, which was used as a control because it is not a heavy metal, was able to induce a significant increase in the levels of AGP mRNA. By 3–6 h postinjection of $HgCl_2$, AGP mRNA levels were 3–4 times higher than in animals treated with Cd, Pb, and Cu and 7–10 times higher than in animals treated with other metals. By 12 h, the Hg- and Cd-induced AGP mRNA levels were equal and were 4–6 times higher than the AGP mRNA levels induced by the other metals.

The same RNA samples were hybridized to the CRP oligonucleotide and MT II probes. The results in Figure 6A,B show that Hg treatment induced the strongest CRP response, which was 3–4-fold greater than the response to the other metals.

Hepatic MT II mRNA levels were also elevated after metal administration. Induction was the highest by Hg and Cd and was maximal by 3 h postinjection for both metals. The other metals, i.e., Zn, Ni, Pb, Cu, and Mg, induced 20–30 times less than Hg and Cd (Figure 7A,B). In all cases, the MT II mRNA levels were back to the control levels by 12 h.

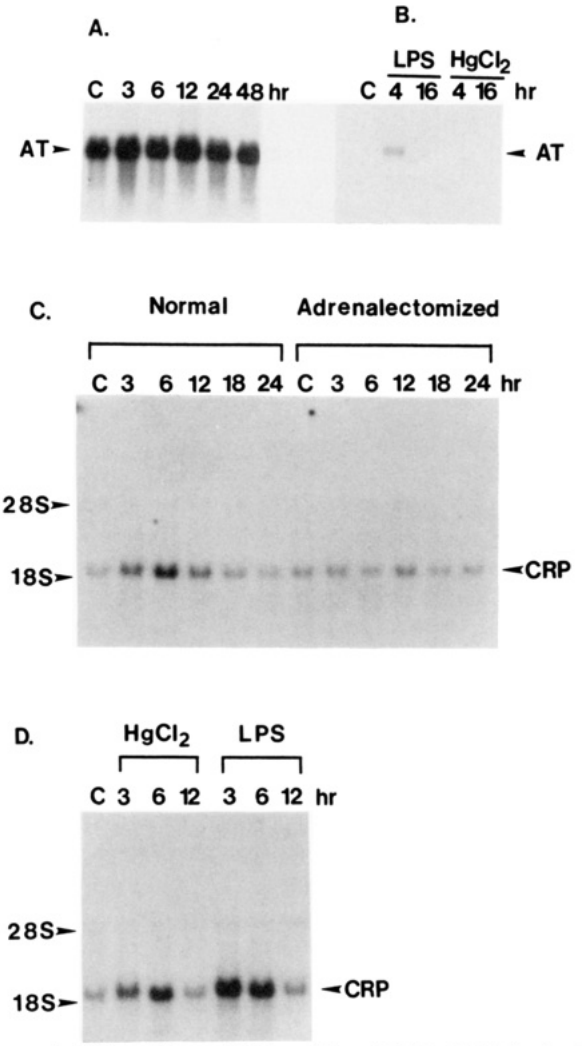

FIGURE 4: Effect of Hg and LPS on AT and CRP mRNA levels. (A) A Northern analysis of AT mRNA levels in the liver of $HgCl_2$ treated mice is shown. A specific AT mRNA probe was prepared by use of the mouse pT7-liv3 cDNA probe. (B) AT mRNA pool levels in peritoneal macrophages were isolated from normal or LPS- or $HgCl_2$-treated mice. Total RNA (5 μg) was separated by agarose gel electrophoresis, transferred to nitrocellulose filters, and subjected to Northern analysis. (C) Accumulation of CRP mRNA in the livers of Hg-treated normal or adrenalectomized mice is shown. (D) The time course shows the increase in CRP mRNA pools in the livers of $HgCl_2$- or LPS-treated mice.

Northern analysis of the RNA samples from the LPS-treated mice and the Hg-treated adrenalectomized mice showed that the time course of induction of MT II gene by $HgCl_2$ is similar to that seen with the LPS-mediated induction. The data also indicate that glucocorticoids are required for maximum expression of the MT II gene, since the MT II mRNA levels in the livers of the Hg-treated adrenalectomized mice were about 5-fold less than in the livers of the nonadrenalectomized mice (Figure 7C). These results suggest that the maximal response of the AGP gene to heavy metals does not require the synergistic action of glucocorticoid as is apparent in the metal-mediated MT II gene expression. None of the metals affected albumin, ACh, AT, or transferrin mRNA levels (data not shown).

Transient Expression Analysis of pAGP(−595)CAT in HepG2 Cells. To determine whether the increase in AGP mRNA levels by heavy metals is due to a mechanism similar to that described for the metallothionein genes, an expression vector, pAGP(−595)CAT, was constructed in which the promoter of the AGP-1 gene, up to −595, was linked to the bacterial chloramphenicol acetytransferase (CAT) gene. The plasmid was transfected into HepG2 cells to determine whether the promoter could drive transcription of the CAT gene in response to metals. The results presented in Figure 8 show that the addition of $HgCl_2$ (10^{-6}–10^{-9} M) to the culture medium resulted in a 3–4-fold increase in CAT activity. The low levels of CAT activity detected in HepG2 cells transfected with pAGP(−595)CAT in the absence of metal are probably due to factors present in the serum or to sequences within the AGP-1 promoter responsible for basal level expression. The above results indicate that the MRE consensus sequences present in the promoter of the AGP-1 gene may be functional. Increased CAT activity was also observed in cell extracts of 10^{-6} M dexamethasone-treated HepG2 cells transfected with pAGP(−595)CAT; however, no synergistic effect was observed when the transfected HepG2 cells were cultured in the presence of various concentrations of $HgCl_2$ and 10^{-6} M dexamethasone (Figure 8B). Similar results were observed with $CdCl_2$ (data not shown). The above in vitro experiments support the observations of the in vivo studies, which suggest that the metal-mediated induction of the AGP gene is not mediated via glucocorticoid interactions.

Discussion

We have demonstrated the induction of mouse AGP and CRP genes by Hg, Cd, Pb, Cu, Zn, Ni, and Mg. Our studies also suggest that there may be a significant difference between the responses of these two genes to the metals because the AGP gene is inducible to maximal levels without the interaction of either glucocorticoids or cytokines, whereas the CRP gene appears to require glucocorticoids for maximal response. In this sense, the CRP gene acts more like the metallothionein gene.

Our studies indicate that the mechanism of regulation of the AGP genes (AGP-1 and AGP-2) by heavy metals is likely to be at the transcriptional level. This is based on previous observations that intermediates of mRNA processing are only detected when transcription is increased during the early phases of LPS-, glucocorticoid-, and cytokine-mediated induction of AGP (Shiels et al., 1987; Carter et al., 1989a). More direct evidence that the response to Hg is transcriptional is provided by the transient expression analyses, which showed that the expression vector pAGP(−595)CAT is inducible in the presence of $HgCl_2$ in the HepG2 human hepatoma cell line.

In previous studies, we demonstrated that the genome of Balb/c mice contains three AGP genes (Cooper & Papaconstantinou, 1986; Cooper et al., 1987). Sequence analysis of one of these, the AGP-1 gene, indicated the presence of four MRE consensus sequences that exhibit homology to mouse MT promoters (Table I) as well as a potential GRE (Cooper et al., 1987). The ability of the pAGP(−595)CAT fusion gene to respond to Hg or dexamethasone in transient expression assays indicates that the MREs and the GRE of the AGP-1 promoter are functional. The AGP-1 MREs do not perfectly match the known mouse MT MREs; however, the conserved core element TGCPuC (Pu = purine) present in the mouse MT MREs (Seguin & Hamer, 1987) is also present in the AGP-1 gene MREs. Hg administration in vivo results in 10–15-fold increase in the liver AGP mRNA levels while in vitro the increase in Hg-mediated CAT gene expression is only 3–4-fold. This may be due to the fact that AGP-2 gene expression is significantly higher than the AGP-1 gene expression in their responses to both LPS (Carter et al., 1991) and heavy metals (Yiangou et al., manuscript in preparation) and that the in vivo response is not directly comparable to that in the HepG2 cells. On the basis of these studies, we propose that

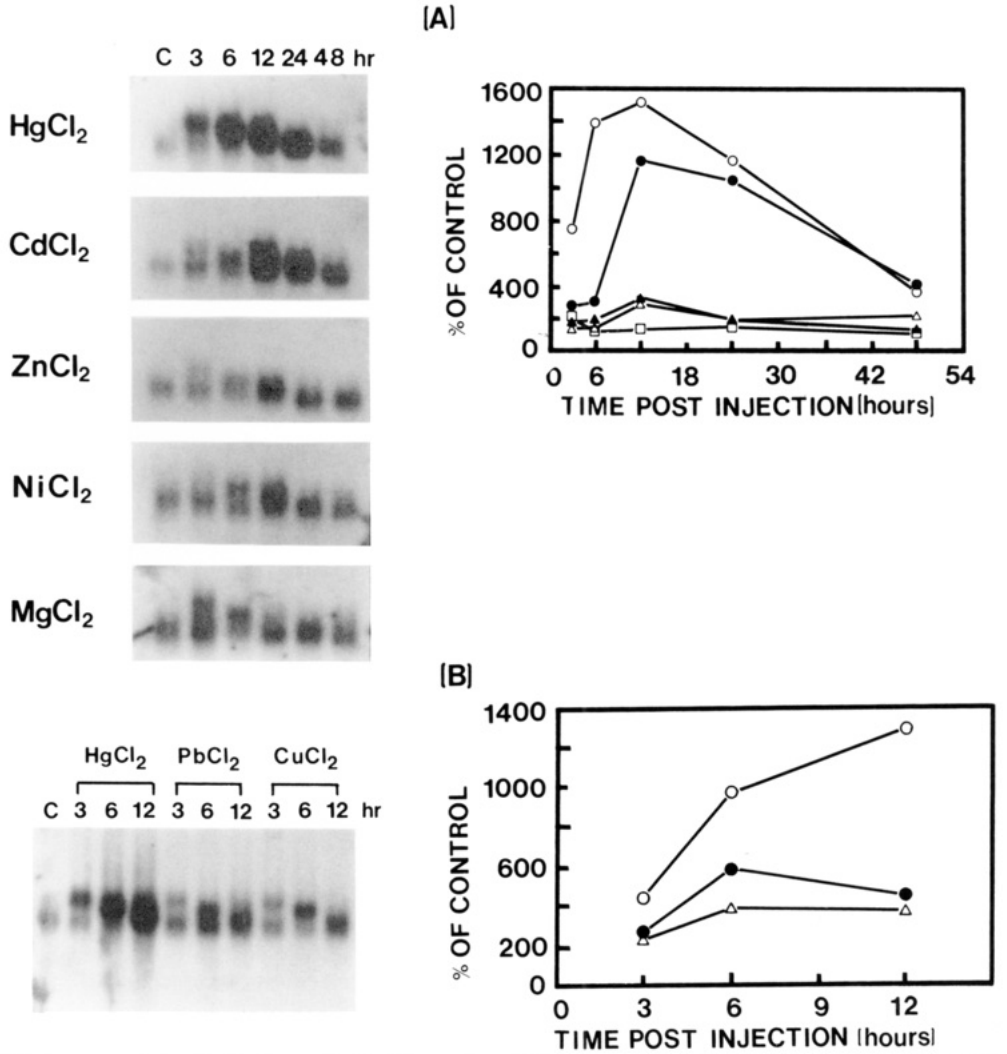

FIGURE 5: AGP mRNA accumulation in the livers of mice injected ip with heavy metals: (A) $HgCl_2$ (○), $CdCl_2$ (●), $ZnCl_2$ (△), $NiCl_2$ (▲), and $MgCl_2$ (□); (B) $HgCl_2$ (○), $PbCl_2$ (●), and $CuCl_2$ (△).

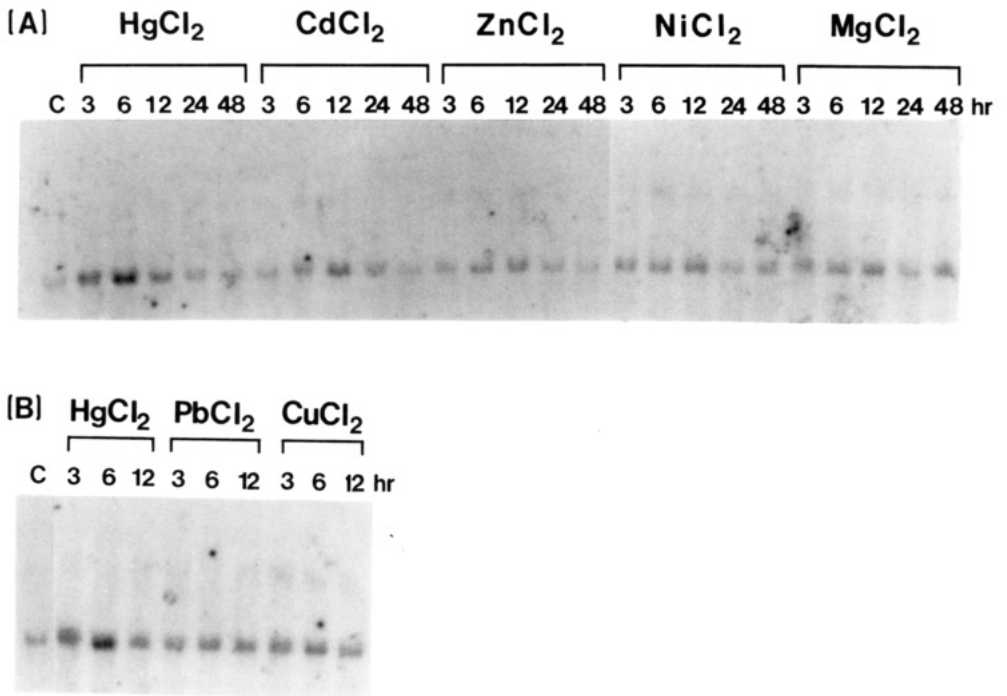

FIGURE 6: Autoradiogram showing the induction of CRP mRNA levels in the livers of heavy-metal-treated mice. RNA samples are the same as those used in Figure 5.

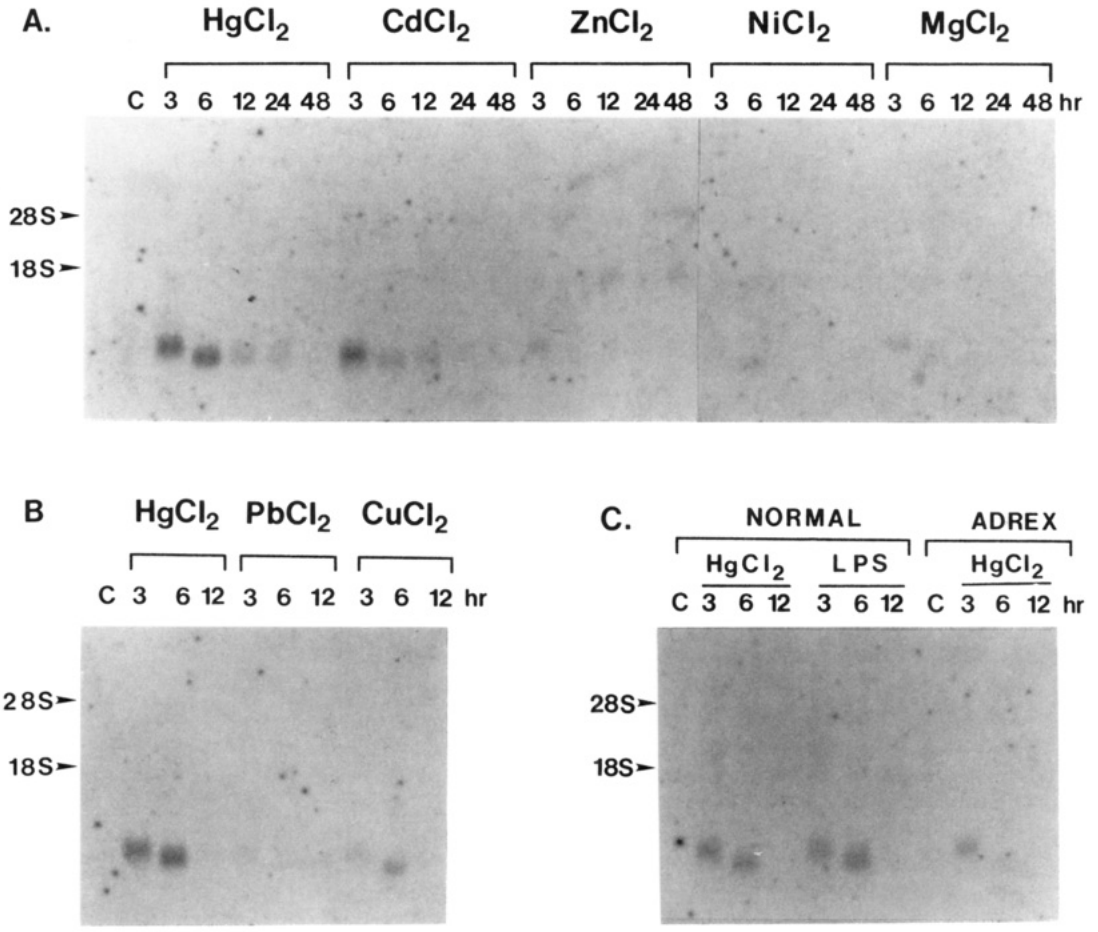

FIGURE 7: Autoradiogram showing the accumulation of MT-II mRNA in the livers of heavy-metal-treated mice. Panels A and B show the effect of heavy metals in MT-II gene expression in the livers of normal mice. RNA samples are the same with those used in Figure 5. Panel C shows the effect of $HgCl_2$ and LPS on MT-II mRNA accumulation in the liver of normal or adrenalectomized mice.

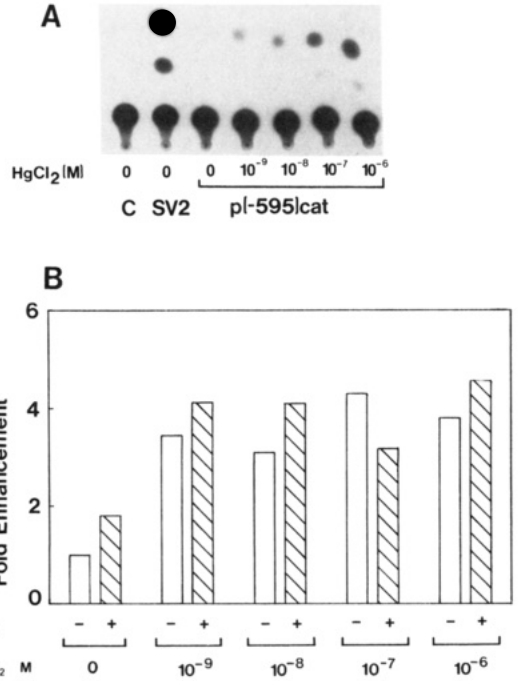

FIGURE 8: Transient expression analysis of pAGP(−595)CAT. (A) The dose–response curve shows the effect of $HgCl_2$ on the expression of pAGP(−595)CAT in HepG2 cells. (B) The histogram shows the effect of $HgCl_2$ and dexamethasone either by itself or in combination on the expression of pAGP(−595)CAT in HepG2 cells.

the mouse AGP gene(s) is inducible by heavy metals independently of either glucocorticoids or cytokines. The induction

Table I: Comparison of the Metal-Responsive Elements (MRE) in the Promoters of the Mouse Metallothionein, AGP, and CRP Genes[f]

Gene	MRE sequence and location
MT MRE CONSENSUS	CTNTGCRCNCGGCCC
MoAGP-1[a]	(+3361)CTGTGCACttTGatt(+3385)
MoMT-I(e)[b]	(-175)CTGTGCACACTGGCG(-161)
MoAGP-1	(-592)CTGTGCACAgaccCt(-578)
MoAGP-1	(-149)CgCTGCACaaaGCtg(-135)
MoMt-I(d)[b]	(-150)CTCTGCACTCCGCCC(-136)
MoAGP-1	(-127)AGAACATTTTGCGCAAGACAT(-108)
MoMt-I(a)[b]	cTTTGCGCccGgacT
MoMT-II(a)[b]	(-58)TTTTGCGCtcGACcc(-44)
MoMT-II(g)[b]	(-297)cTTTGCGCtcagCtC(-283)
MT MRE[c] CONSENSUS	CYTTTGCRYYCG
MOCRP	(-211)tTTTTGCtTCCc(-200)
	(-181)CgTTTaCcCCaa(-170)
MoMT-II C[d]	CCnGGGCGG
MoCRP[e]	(-88)CCGaGGCaG(-80)
MoMT-II D[d]	CGCGGGGCGnGTGCA
MoCRP[e]	(-95)gctGGGcCcGagGCA(-79)

[a] Cooper et al. (1987). [b] Stuart et al. (1985). [c] Stuart et al. (1984). [d] Searle et al. (1984). [e] Ohnishi et al. (1988). [f] n = A, T, G, or C; R = A or G; Y T or C.

of metallothionein I by heavy metals, such as Cd and Cu, is mediated by the direct interaction of a trans-acting factor with the cis-acting MREs (Seguin et al., 1984; Seguin & Hamer, 1987; Seguin & Provost, 1988; Westin & Schaffner, 1988; Imbert et al., 1989). We believe that a similar mechanism may be involved in the Hg-mediated induction of the mouse AGP gene(s). Furthermore, we propose that this is a new pathway for the regulation of the AGP and CRP genes in the mouse.

Although the AGP genes are induced by the same heavy metals that induce MT, there may be significant differences between these reactions. For example, the induction of MT is most efficient in the presence of glucocorticoid, indicating a cooperative effect by the MREs and GRE trans-acting factors. Hg appears to act as a potent inducer of AGP in the absence of glucocorticoids. This may be due to the promoter structure. For example, in the AGP-1 promoter there is a close alignment of two MRE sequences and one GRE consensus sequence (for −149 to 108) that may prevent simultaneous binding to all three sites by their respective trans-acting factors (Cooper et al., 1987). The MT II promoter, however, shows scattered MREs that may facilitate a cooperative action by these trans-acting factors. The above interpretation is supported by the transient expression analyses in which the addition of dexamethasone to Hg-treated HepG2 cells does not mediate a significant increase in expression of pAGP(−595)-CAT in HepG2 cells, that is, above that seen with Hg alone.

There is the possibility that the injection of Hg and other metals results in an inflammatory response and subsequent increased levels of glucocorticoid and cytokines, such as IL 6. We believe it unlikely that the low concentration of Hg necessary to induce the expression of these genes could cause an increase in cytokine or glucocorticoid levels to the extent that they could induce the high levels of AGP mRNA we observed. Furthermore, cytokines such as IL 6 mediate the negative regulation of albumin gene expression and a corresponding reduction of the albumin mRNA pool (Darlington et al., 1986; Perlmutter et al., 1986; Gauldie et al., 1987; Andus et al., 1988a,b; Geiger et al., 1988a,b; Carter et al., 1989b; Prowse & Baumann, 1989). Our Northern analyses consistently showed that albumin mRNA levels are not reduced by any of the heavy metals that induce AGP mRNA and are in agreement with the results of Piscator et al. (1970), who showed no effect on albumin serum levels in rabbits chronically exposed to Cd. In addition, we observed that AT mRNA levels are increased in peritoneal macrophages during an LPS-mediated inflammatory response, confirming that the macrophages elicit an acute-phase response with LPS (Perlmutter & Punsal, 1988). Hg treatment on the other hand has no effect (Figure 4A), which indicates a specific response to heavy metals occurs in the liver in the absence of an acute-phase response. Our data show that Hg-mediated MT II induction in adrenalectomized mice is significantly lower than in the intact mice; these results agree with previous studies (Yagle & Palmiter, 1985) showing that metals and glucocorticoids have a cooperative effect on hepatic MT gene expression and suggest that Hg administration does not initiate an inflammatory response.

The strongest induction of AGP was mediated by Hg, which was followed in order of response by Cd > Pb > Cu > Zn > Ni and Mg. These data suggest that one of the functions of this protein may be to bind toxic metals as well as to protect against increased levels of essential metals such as Zn, Cu, and Mg, which are released into the circulation as a result of cellular damage. Metallothionein is a major -SH-containing protein whose function is to bind heavy metals such as Cd. Examination of the AGP-1 and AGP-2 protein sequences derived from their cDNA and genomic sequences (Cooper & Papacontantinou, 1986; Cooper et al., 1987) indicates that neither of these proteins is a sulfhydryl protein; i.e., they contain only two cysteines, one in exon 3 and one in exon 6. In view of this, specific binding studies must be done to determine if AGP can function as a heavy-metal scavenger.

In our survey to determine whether other members of the APR family of genes are inducible by heavy metals, we found that CRP is also inducible by Hg. As with AGP, analysis of the CRP gene revealed sequences located in its proximal promoter that exhibit homology to the MT MREs (Table I). Whether they are functional MREs has not been determined. Furthermore, this induction occurs at a very low level in adrenalectomized animals, indicating that, as with the MTs, glucocorticoids may be required for a maximal induction of this gene.

Eukaryotic organisms produce APRs, MTs, and HS proteins in response to various stress stimuli. The studies presented here have shown that at least two of the members of the APR family of genes are induced by heavy metals and that one of them, the AGP gene, is induced by a mechanism independent of the glucocorticoid or cytokine pathways.

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