

Identification of a *cis*-Acting Element That Is Responsible for Cadmium-mediated Induction of the Human Heme Oxygenase Gene*

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Kazuhisa Takeda‡, Shinobu Ishizawa‡, Michihiko Sato§, Tadashi Yoshida¶, and Shigeki Shibahara‡||

From the ‡Department of Applied Physiology and Molecular Biology, Tohoku University School of Medicine, Sendai, Miyagi 980-77, the §Central Laboratory for Research and Education and the ¶Department of Biochemistry, Yamagata University School of Medicine, Yamagata 990-23, Japan

Heme oxygenase is an essential enzyme in heme catabolism and is inducible by various environmental rearrangements such as cadmium. The activity and mRNA levels of heme oxygenase were remarkably increased in HeLa cells by the treatment with cadmium. As a first step in studying the molecular mechanisms of this induction, we performed transient expression assays in four human cell lines including HeLa to analyze the cadmium-mediated inducibility of the fusion genes, containing the firefly luciferase gene as a reporter under the human heme oxygenase gene promoter. By determining the luciferase activity expressed in the transfected cells, we found the region between about 4.5 and 4 kilobase pairs upstream from the transcriptional initiation site of the heme oxygenase gene which confers cadmium-mediated inducibility on the fusion gene. The region was then subjected to further functional analysis in HeLa cells, which allowed us to localize the cadmium-responsive element to 20 base pairs. Gel mobility shift assays demonstrated that this 20-base pair element is specifically bound by nuclear protein(s) of HeLa cells, the binding activities of which were however unchanged by the treatment with cadmium. Using the synthetic cadmium-responsive elements containing various base changes, we have identified a 10-base pair sequence, TGCTAGATT, required for the cadmium-mediated inducibility and *in vitro* protein binding. We thus suggest that this binding protein(s) is involved in the cadmium-mediated activation of the heme oxygenase gene. Incidentally, the consensus sequence of AP-1 binding site, TGAGTCA, is present downstream of this cadmium-responsive element. However, we provide evidence that AP-1 is not directly involved in the cadmium-mediated induction of the human heme oxygenase gene.

Heme oxygenase (EC 1.14.99.3) is an essential enzyme in heme catabolism. It cleaves heme to form biliverdin (1), which is subsequently converted to bilirubin by biliverdin reductase (2). Heme oxygenase is ubiquitously expressed in animal cells,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D17675.

|| To whom correspondence should be addressed: Dept. of Applied Physiology and Molecular Biology, Tohoku University School of Medicine, Aoba-ku, Sendai, Miyagi 980-77, Japan. Fax: 81-22-272-7273.

and its activity is highest in the spleen, where senescent erythrocytes are sequestered and destroyed (3). Recently it was suggested that heme oxygenase also functions as a defense system against oxidative stress, since biliverdin or bilirubin produced locally may work as a physiological antioxidant (4). Indeed, heme oxygenase activity is highly inducible by its substrate heme (4–8), heme-hemopexin (9), heavy metals (10, 11), bromobenzene (12), interleukin-6 (13), ultraviolet light (14), and heat shock (15). The induction of heme oxygenase by hemin or cadmium is observed in all animal cells examined (4–8, 10, 11), suggesting that this induction is of physiological significance. It was already shown that hemin or cadmium increased the transcription of the heme oxygenase gene (16). However, in contrast to heme/hemin, a biological function of cadmium has not been defined yet, although cadmium is present in minute traces in living organisms. Because cadmium is able to bind various cellular components such as free sulfhydryl groups and nucleic acids (for review see Ref. 17), cadmium may simply mimic or modify a physiological mediator that is involved in a signal transduction system leading to activation of the heme oxygenase gene transcription. It is well known that cadmium activates transcription of the metallothionein gene (18). Metallothionein contains an exceptionally large number of cysteine residues and thus efficiently binds cadmium through sulfhydryl groups to reduce its toxicity. It is of significance to clarify whether the mechanism of cadmium-mediated induction of the human heme oxygenase gene is the same as that of the metallothionein gene. In contrast to metallothionein, however, human and rat heme oxygenase proteins lack a cysteine residue (19, 20), suggesting that heme oxygenase protein itself is not directly involved in the detoxification of cadmium. Thus, the physiological relevance of the cadmium-mediated induction of heme oxygenase is still not known. Understanding the molecular mechanism of the cadmium-mediated induction will help us to reveal its physiological implication.

Recently an isozyme of heme oxygenase was identified and referred to as heme oxygenase-2 by Maines's group (21–24). The amino acid sequence of heme oxygenase-2 (23, 24) shares 40% similarity with that of the authentic heme oxygenase (19, 20), designated as heme oxygenase-1 (21). Each heme oxygenase isozyme is encoded by a separate gene, and recently the human heme oxygenase-1 gene has been mapped to chromosome 22q12 (25).

In this study we showed that heme oxygenase-2 is not induced in HeLa human cervical cancer cells by the treatment with hemin or cadmium, whereas authentic heme oxygenase is highly induced. Thus, unless otherwise indicated, the term *heme oxygenase* used in this report represents the authentic heme oxygenase (heme oxygenase-1). We have identified the

10-bp¹ *cis*-acting element responsible for cadmium-mediated induction about 4 kb upstream from the transcription initiation site of the human heme oxygenase gene. Furthermore, we found that nuclear proteins specifically bind to this cadmium-responsive element.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases were purchased from Takara Shuzo, Boehringer Mannheim, and New England Biolabs. Klenow enzyme, T4 DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were from Takara Shuzo; [α -³²P]dCTP, [α -³²P]CTP, and [α -³²P]UTP were from Amersham Corp. [γ -³²P]ATP was from ICN.

Enzyme Assay of Heme Oxygenase—HeLa human cervical cancer cells were maintained in minimum essential medium (MEM) containing 5% fetal calf serum. For induction of heme oxygenase, cells were cultivated for 5 h in serum-free MEM containing 5 μ M hemin or 5 μ M cadmium chloride. Enzyme assay was carried out as described previously (8).

Northern Blotting Analysis—HeLa cells were cultivated for 3 h in serum-free MEM containing 5 μ M hemin or 5 μ M cadmium chloride. Total RNA, prepared by the method of Chirgwin *et al.* (26), was separated in an agarose gel containing 1 M formaldehyde, transferred to a nitrocellulose membrane (Schleicher & Schuell), and hybridized with ³²P-labeled probes (19, 20). The hybridization probes used were the *Xba*I/*Xba*I fragment (-64/923) derived from the human heme oxygenase cDNA, pHHO1 (20), the *Hinf*I/*Hinf*I fragment (38/917) from the human heme oxygenase-2 cDNA, pHHO2-1 (27), and the *Sma*I/*Sca*I fragment (124/1050) from a full-length cDNA for human β -actin provided by Tokuo Yamamoto (Gene Research Center, Tohoku University). The nucleotide residues of the β -actin cDNA were numbered according to the published sequences (28). These probes were labeled with [α -³²P]dCTP by random priming method, and the signals representing the hybridized probes were detected by autoradiography.

Nuclear Run-on Transcription Assay—Intact nuclei were isolated from untreated or cadmium-treated (5 μ M for 0.5, 1.5, and 3 h) HeLa cells and subjected to nuclear run-on transcription as described previously (29). The DNA fragments blotted onto a nitrocellulose membrane were the linearized pBluescript II SK(+) (Stratagene), carrying the cDNA coding for either heme oxygenase-1, heme oxygenase-2, or β -actin.

Cloning of Genomic DNA Containing the 5'-Flanking Region of the Human Heme Oxygenase Gene—A genomic DNA library, constructed in EMBL3 using *Sau*3AI partial digests of human lymphocyte DNA, was screened by *in situ* plaque hybridization. The hybridization probe used was the *Pvu*II/*Hinf*I fragment (-58/+221) derived from λ HHO9 (30), containing the promoter region and exon 1 of the human heme oxygenase gene. One clone, containing the 5'-flanking region of the human heme oxygenase gene, was isolated and named as λ HHO12 (see Fig. 3).

Plasmid Construction—pSV2/L (31), containing the luciferase gene under the SV40 early promoter, was kindly provided by S. Subramani (University of California at San Diego, La Jolla, CA). The *Hind*III/*Sma*I fragment containing the entire luciferase gene was isolated from pSV2/L and was subcloned into the *Hind*III/*Sma*I sites between *Xba*I and *Bam*HI sites in the polylinker sequence of pBluescript II SK(+) (Stratagene). Then the *Xba*I/*Bam*HI fragment containing the luciferase gene was isolated and ligated to pHHOgpt2 (20) or other pHHOgpt constructs linearized with *Xba*I and *Bam*HI to replace the *gpt* gene with the luciferase gene. The pHOL constructs thus obtained carry the luciferase gene under the human heme oxygenase gene promoter. The 5' end of the promoter region is the *Hind*III site or a blunt end, and its 3' end is the *Xba*I site. For example, pHHOL14 and pHHOL5 contain the *Hind*III (*Xba*I)/*Xba*I fragment (about -4 kb/+20) and the *Hind*III (*Pst*I)/*Xba*I fragment (-282/+20), respectively, the *Xba*I and *Pst*I sites of which were converted to the *Hind*III sites. The restriction sites shown within the parentheses represent the original sequence found in the gene. pHHOL15 contains the *Pst*I/*Xba*I fragment (about -4.5 kb/+20), the *Pst*I site of which was converted to a blunt end. The fragments used for the promoter region of these pHOL constructs were derived from λ HHO9 (30) or λ HHO12 (see Fig. 3A). A promoterless construct, pHHOL0, constructed by self-ligating the pHHOL5 linearized with *Hind*III, contains no heme oxygenase gene promoter upstream of the luciferase gene (see Fig. 3B). For localization of a cadmium-responsive element, the five fragments located between -4.5 kb/-4 kb, the *Pst*I/*Xba*I, the *Pst*I/*Pvu*II, the *Nar*I/*Xba*I, the *Hinf*I/*Hinf*I and the *Hinf*I/*Xba*I

fragments were isolated and ligated to the pHHOL5 linearized with *Hind*III, yielding to five fusion plasmids, pHHOL20, pHHOL22, pHHOL23, pHHOL25, pHHOL26 (see Fig. 6). Prior to ligation, the *Hind*III site (originally the *Pst*I site) of the pHHOL5 and the ends of each fragment were converted to blunt ends.

Synthetic double-stranded oligonucleotides, Cd1, Cd2, Cd4, and Cd5, were designed to localize the minimum sequence required for cadmium-mediated induction (see Fig. 6A). For convenience in plasmid construction, each element contains additional sequences at both ends to create the restriction sites; namely, each element contains the TCGAG residues at the 5' end and the G residue at the 3' end in the sense strand and contains the TCGAC residues at the 5' end and the C residue at the 3' end in the antisense strand (see Fig. 8A). The cohesive end, TCGA, is common to the cleavage sites of both *Xba*I and *Sal*I, and each element thus contains the linker sequences for the *Xba*I cleavage site at the 5' end and for the *Sal*I cleavage site at the 3' end. These elements were inserted into the *Sal*I site of pHHOL5, which had been created from the native *Pst*I site, yielding four fusion genes, pHHOLcd1, 2, 4, and 5. In the same way, six fusion genes carrying the mutant oligonucleotides, MT1, MT2, MT3, MT4, MT5, and MT6, were constructed (see Fig. 8A). For the functional analysis using a heterologous promoter, the *Bam*HI (*Eco*RI)/*Hind*III fragment containing the SV40 promoter was isolated from pCAT™-Promoter plasmid (Promega) and was inserted between the *Bam*HI and *Hind*III sites of pHTRPSVL1 (32) to replace the tyrosinase-related protein gene promoter with the SV40 promoter. pSVLE(-) thus obtained carries the SV40 promoter upstream from the luciferase gene. The oligonucleotide Cd5 was inserted between the *Bam*HI and *Bgl*II sites of pSVLE(-), located upstream from the SV40 promoter, in the sense (pSVLCd5) and antisense direction (pSVLCd5i) by blunt end ligation (see Fig. 7).

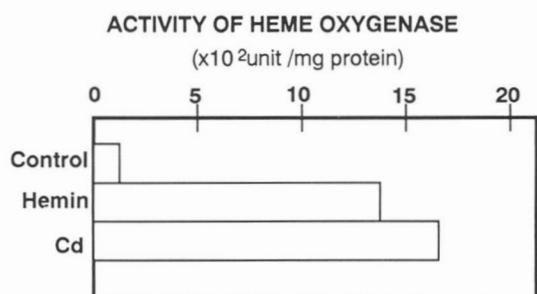
Transient Expression Analysis of Fusion Genes—HeLa cells, about 70% confluent in 6-cm dishes, were transfected by the calcium phosphate method (31, 33, 34) with modifications; namely, cells were incubated with the plasmid DNA precipitated with calcium phosphate for 16 h, refed with fresh medium, and incubated for 24 h. The DNA used for cotransfection was 7 μ g of each fusion gene and 1 μ g of β -galactosidase expression vector pCH110 (Pharmacia Biotech Inc.), containing the SV40 early promoter, as an internal control. Following the 24-h incubation, cells were incubated with 5 μ M CdCl₂, hemin, sodium arsenite, or cobalt protoporphyrin in serum-free MEM. After incubation for 5 h, cells were lysed by repeated freezing and thawing in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol, and centrifuged 10,000 \times g for 10 min. The supernatants were assayed for luciferase activity (31) and for β -galactosidase activity (35) as an internal control. Luciferase activity was divided by β -galactosidase activity and was shown as a ratio to the value obtained with pHHOL5 to calculate the relative activity. For some experiments, MeWo human melanoma cells were similarly transfected as described above, and YN-1-0-A human erythroleukemia cells and THP-1 human myelomonocytic leukemia cells were transfected by the DEAE-dextran method (36). MeWo, YN-1-0-A, and THP-1 cells were cultivated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, in serum-free Iscove's modified Dulbecco's medium supplemented with 2 μ M FeCl₃ (27) and in RPMI 1640 with 10% fetal calf serum, respectively. Only THP-1 cells were treated with 50 μ M cadmium in the presence of 10% fetal calf serum. In all cases, the data shown are the mean \pm S.D. of at least three independent experiments.

RNase Protection Assay—HeLa cells were transfected with pHHOL14 or pHHOL15 in the same way as described above and treated for 3 h with 5 μ M cadmium. Total RNA was subjected to RNase protection assay. The riboprobe was prepared from a subclone, carrying the *Pvu*II/*Asu*II fragment derived from pHHOL5 in the *Sma*I site of pBluescript II SK(+). The *Pvu*II site is located 60 bp upstream from the transcription initiation site, and the *Asu*II site is located within the luciferase cDNA. The *Asu*II site was converted to a blunt end prior to ligation. Riboprobe was synthesized in the opposite direction of pHHOL transcription using T7 promoter and labeled with [α -³²P]CTP (see Fig. 3C). RNase protection assays were performed according to the method of Melton *et al.* (37). RNA was hybridized with the riboprobe at 42 °C overnight, digested with RNase mixture (Ambion) containing RNase A1 (1 mg/ml) and RNase T1 (20,000 units/ml), then incubated with proteinase K, and precipitated with ethanol. The protected RNA was separated on a 5% polyacrylamide gel containing 7 M urea.

Nuclear Extract Preparation—Nuclear extracts were prepared by the modified method of Lassar *et al.* (38). HeLa cells, seeded in 15-cm-diameter dishes, were incubated in serum-free MEM containing 5 μ M cadmium or no addition for 90 min. Cells were washed three times with phosphate-buffered saline and lysed with 2.5 ml of the lysis buffer (20

¹ The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); MEM, minimum essential medium; TPA, 12-O-tetradecanoylphorbol-13-acetate.

A



B

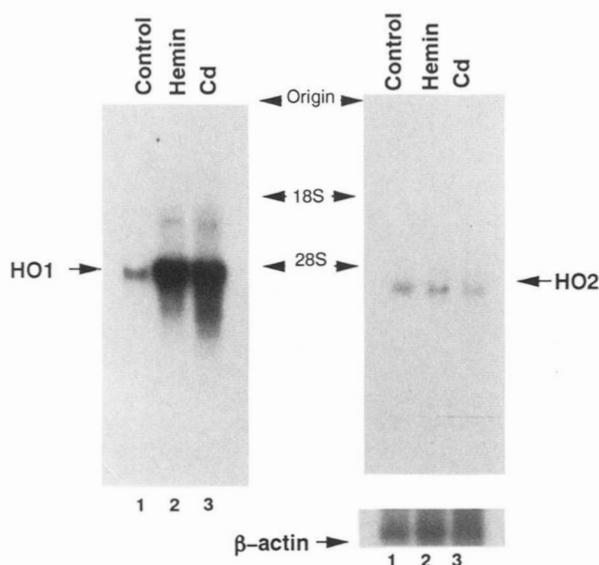


FIG. 1. Induction of heme oxygenase in HeLa cells. Panel A, induction of heme oxygenase activity. HeLa cells were either untreated or treated for 5 h with 5 μ M hemin or cadmium. The specific activity of heme oxygenase was multiplied by 100 and shown. Panel B, induction of heme oxygenase mRNA. HeLa cells were incubated for 3 h under the indicated conditions. The relative changes in the amounts of heme oxygenase-1 (*HO1*) and heme oxygenase-2 (*HO2*) mRNAs were determined by Northern blotting analysis. Heme oxygenase-1 mRNA levels are shown to the left and heme oxygenase-2 mRNA levels to the right. Each lane contained 10 μ g of total RNA prepared from the cells treated with no additions (lane 1), 5 μ M hemin (lane 2), or 5 μ M cadmium (lane 3). The size markers were human rRNA. The β -actin mRNA levels are shown at the bottom as an internal control, indicating the variability in the amounts of RNA loaded.

mm HEPES (pH 7.9), 20% glycerol, 10 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride) was added. Nuclei were pelleted by centrifugation for 5 min at 2,000 rpm at 4 °C and resuspended at 2.5×10^7 nuclei/ml in the lysis buffer containing 400 mM KCl, gently rocked for 1 h at 4 °C, centrifuged at 10,000 rpm for 10 min. The resulting supernatant was dialyzed against the buffer (20 mM HEPES (pH 7.9), 100 mM KCl, 20% glycerol, 0.5 mM dithiothreitol, and 0.5 mM phenylmethanesulfonyl fluoride) and used as nuclear extracts. The nuclear extracts were quickly frozen in liquid nitrogen and stored at -80 °C.

Gel Mobility Shift Assay—Nuclear extracts were incubated with 0.5–1 ng of ³²P-labeled probe (5,000–10,000 cpm) in a reaction buffer containing 20 mM HEPES (pH 7.9), 5% glycerol, 50 mM KCl, 1.5 mM MgCl₂, 1.2 μ g of poly(dI-dC), either with or without competitors. The probe used was the double-stranded oligonucleotide Cd5 or Cd2, and the competitors used were the synthetic oligonucleotides (Cd1, Cd2, Cd4, and Cd5), AP-1 consensus oligonucleotide (Promega), or mutant oligonucleotides (MT1, MT2, MT3, MT4, MT5, and MT6). After a 30-min incubation at room temperature, the reaction buffer was loaded onto 4% polyacrylamide gels containing 6.7 mM Tris-HCl (pH 7.9), 3.3 mM acetic acid, and 1 mM EDTA and electrophoresed at 150 V for 3 h at 4 °C.

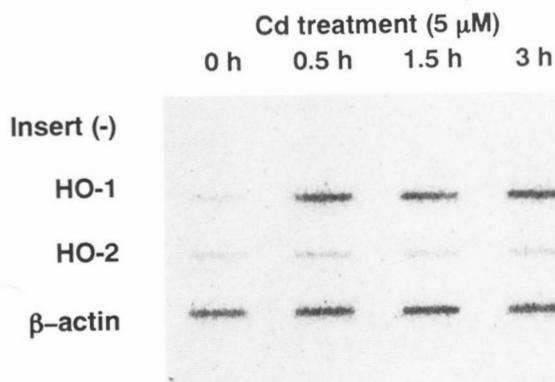


FIG. 2. Transcriptional activation of the heme oxygenase gene by cadmium. Nuclear run-on transcription was performed with nuclei isolated from the HeLa cells untreated or cadmium treated for the indicated time. Each ³²P-labeled RNA was hybridized to a nitrocellulose membrane, which was slot blotted with denatured plasmids containing no inserts or human cDNA for heme oxygenase-1 (*HO-1*), heme oxygenase-2 (*HO-2*) or β -actin.

RESULTS

Induction of Heme Oxygenase by Cadmium—We initially confirmed the effects of hemin or cadmium on expression of heme oxygenase activity in HeLa cells. Both hemin and cadmium increased the heme oxygenase activity by about 10-fold (Fig. 1A). The concentration of hemin or cadmium (5 μ M) did not affect the cell viability (data not shown). We then analyzed the effects of hemin or cadmium on the expression of two heme oxygenase mRNAs in HeLa cells. Heme oxygenase-1 mRNA was induced by treatment with hemin or cadmium (Fig. 1B), whereas heme oxygenase-2 mRNA was not induced by treatment with hemin or cadmium. These results are consistent with our previous report that human heme oxygenase-2 is constitutively expressed but is not induced under the conditions in which heme oxygenase-1 is highly induced (27). To confirm that cadmium acts at the transcriptional levels to induce heme oxygenase-1, a nuclear run-on transcription assay was performed with isolated nuclei (Fig. 2). Consistent with the result of Northern blot analysis, heme oxygenase-1 transcription was remarkably activated at 0.5 h of treatment with cadmium, whereas the level of heme oxygenase-2 or β -actin transcription was not activated. Thus, in this report we focused on the heme oxygenase-1 gene to study the molecular mechanisms of its transcriptional activation by cadmium.

Cloning of Genomic DNA Containing the 5'-Flanking Region of the Human Heme Oxygenase Gene—We have shown that the 5'-flanking region of the human heme oxygenase gene, containing about 1.4 kb upstream from the transcription initiation site, is unable to confer the induction of the reporter gene by hemin (30) or cadmium (data not shown). We therefore isolated a new clone, λ HHO12 (Fig. 3A), which contains the 5'-flanking region of about 18 kb and exon 1 of the heme oxygenase gene. Using blot hybridization analysis of human placenta DNA, we confirmed that the cloned heme oxygenase gene retains the same sequence organization as in the human genomic DNA (data not shown), excluding a possibility of cloning artifacts. We thus prepared the 5'-flanking region of the heme oxygenase gene from λ HHO12 and used this DNA as a promoter region for construction of fusion plasmids.

Presence of a cis-Acting Element Responsible for Cadmium-mediated Induction of the Heme Oxygenase Gene—To help define the DNA segment responsible for the cadmium-mediated induction of the heme oxygenase gene, its promoter function was analyzed by transient expression of various pHOL constructs, containing the 5'-flanking region of the heme oxygen-

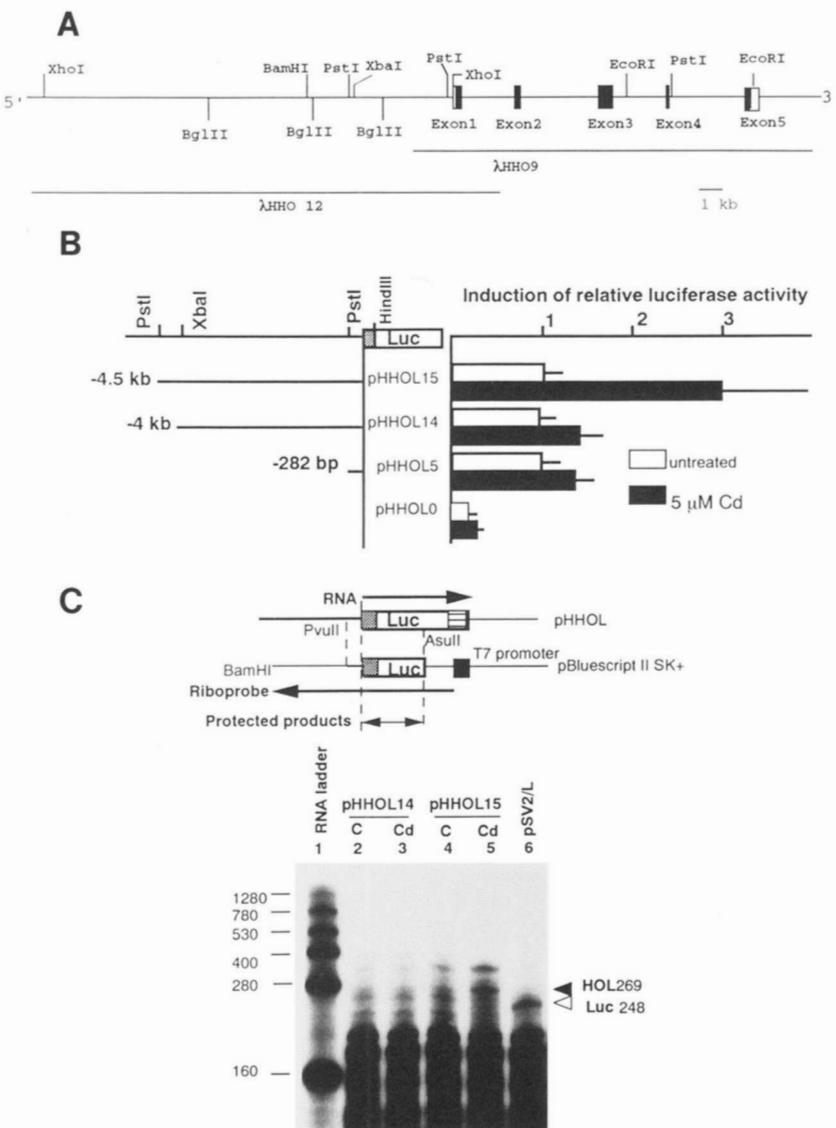


FIG. 3. Presence of a cadmium-responsive element in the 5'-flanking region of the human heme oxygenase gene. *Panel A*, restriction map of the cloned genomic DNA encoding human heme oxygenase. The direction of transcription is from left to right. The sites for relevant restriction enzymes are shown. The locations of the exons are indicated by open boxes (untranslated regions) and by closed boxes (protein-coding regions). *Panel B*, presence of a cadmium-responsive element. HeLa cells were cotransfected with β-galactosidase expression vector pCH110 and each fusion gene indicated. The promoter regions used for construction of fusion genes are schematically shown to the left. The exon 1 coding for the 5'-untranslated region of the heme oxygenase gene is indicated by a stippled box. The HindIII site is located in the 5'-flanking region of the luciferase gene (31). The numbers at the 5' end of each construct represent the positions from the transcription initiation site of the heme oxygenase gene. Transfected cells were incubated with or without 5 μM cadmium for 5 h and were harvested for the assays of luciferase activity and β-galactosidase activity. The luciferase activity was divided by β-galactosidase activity (as an internal control) to correct the variability in transfection efficiency and is shown as a ratio to the value obtained with pHHOL5 as described under "Experimental Procedures." The relative luciferase activity is shown to the right. The data shown are the mean ± S.D. of five independent experiments. *Panel C*, transcriptional activation of the fusion gene by cadmium treatment. HeLa cells transfected with pHHOL14 (lanes 2 and 3), pHHOL15 (lanes 4 and 5), or pSV2L (lane 6) were cultivated for 3 h in serum-free MEM with no additions (lanes 2, 4, and 6) or 5 μM cadmium (lanes 3 and 5). RNase protection assay was performed as described under "Experimental Procedures." The expected size of the protected transcripts is 269 nucleotides (indicated by a closed arrowhead). The transcripts of pSV2L, a positive control, are shown with an open arrowhead. The size marker (lane 1) used was the RNA ladder purchased from Life Technologies, Inc., and relevant sizes are given in nucleotides.

ase gene upstream from the luciferase gene (Fig. 3B). Cadmium treatment increased the relative luciferase activity by about 3-fold in the cells transfected with pHHOL15 containing the 5'-flanking region of about 4.5 kb. This increase is significantly higher than the value obtained with pHHOL5. In contrast, the relative activity obtained with pHHOL14, containing the 5'-flanking region of about 4 kb, is not significantly higher than the value with pHHOL5. A promoterless construct, pHHOL0, gave rise to low but detectable luciferase activity, which was, however, not induced by cadmium. Furthermore, we have confirmed that cadmium treatment has no apparent effects on the luciferase or β-galactosidase activity in the cells

transfected with pSV2L or pCH110, a β-galactosidase expression vector (data not shown). These results suggest that the cadmium-responsive element is located within the *PstI/XbaI* segment (-4.5 kb/-4 kb).

To confirm whether this increase in the relative luciferase activity is caused by transcriptional activation, we performed RNase protection assays (Fig. 3C). We included RNA prepared from the cells transfected with pSV2L as a positive control (lane 6). The protected products of about 270 nucleotides represent the transcripts correctly initiated from the pHOL constructs, whereas the expected size of pSV2L transcripts was about 250 nucleotides (lane 6). Cadmium treatment induced

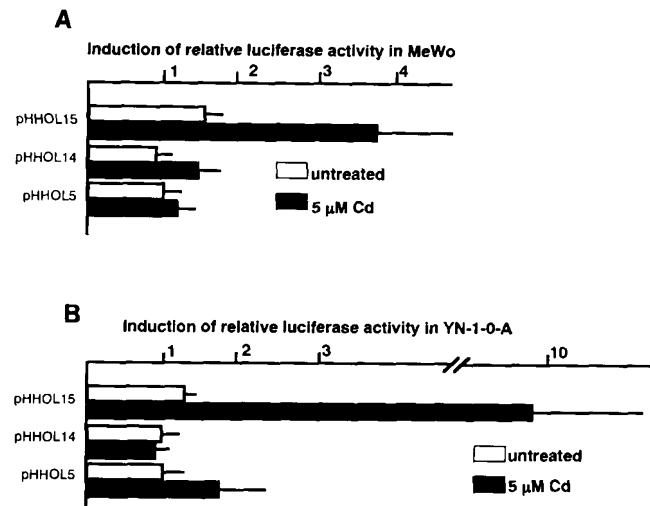


FIG. 4. Functional analysis of the cadmium-responsive element of the human heme oxygenase gene in other human cells. Transient expression of the fusion genes was performed in MeWo melanoma cells (*panel A*) and YN-1-0-A leukemia cells (*panel B*). The experimental conditions were the same as described in the legend to Fig. 3B.

the expression of pHOL15 by about 3-fold (*lanes 4 and 5*) but did not induce the expression of pHOL14 (*lanes 2 and 3*). This increase in the amounts of the transcripts was consistent with that of the luciferase activity. These results indicate that the increase in luciferase activity detected in the cells transfected with pHOL15 is mainly caused by transcriptional activation.

Cadmium-mediated induction of heme oxygenase is observed in various human cell lines examined (14, 39). Thus, to confirm that the cadmium-mediated activation of the fusion gene expression seen in HeLa cells is a general phenomenon, we performed similar assays using two other human cell lines of different cell lineages, MeWo melanoma and YN-1-0-A leukemia cells. In these cell lines, heme oxygenase mRNA is remarkably induced by either hemin or cadmium as seen in HeLa cells (data not shown). As shown in Fig. 4, the treatment with cadmium increased the relative luciferase activity about 3-fold in MeWo and about 10-fold in YN-1-0-A cells transfected with pHOL15 but not with pHOL14. These results also support that the cadmium-responsive element is present in the DNA segment carried by pHOL15 but is deleted in pHOL14. Consequently, the *PstI/XbaI* segment (-4.5 kb/-4 kb), carried by pHOL15, was sequenced and shown in Fig. 5A. We found that the sequence shares about 64% similarity with that of the region responsible for the TPA-mediated induction of the mouse heme oxygenase gene (40). Incidentally, a TGAGTCA motif, a consensus sequence of AP-1 binding site, is present near the 3' end of this cadmium-responsive region, although heme oxygenase is not induced by TPA in HeLa cells (29). In addition, both sequences are located at similar positions, about 4 kb upstream from each transcription initiation site.

Localization of the Cadmium-responsive Element—To localize the cadmium-responsive element within the *PstI/XbaI* fragment, we constructed five plasmids containing the internal deletion of about 4 kb as shown in Fig. 5B. For example, pHOL20 was constructed by ligating this *PstI/XbaI* fragment to the 5' end of the promoter region carried by pHOL5. Cadmium treatment increased the relative luciferase activity by about 2–3-fold in the cells transfected with pHOL20, which is consistent with the magnitude of increase obtained with pHOL15 (see Fig. 3B). Furthermore, the basal level of its expression was not changed compared with that of pHOL5. These results indicate that the deletion of 4 kb does not affect

the inducibility by cadmium nor the basal level of its expression. Consequently, the *PstI/XbaI* fragment was dissected into four fragments, and the function of each fragment was similarly analyzed. The cadmium-mediated induction of relative luciferase activity was observed in the cells transfected with pHOL23 or pHOL25. These increases were significantly higher than the values with pHOL22, pHOL26, or pHOL5. These results suggest that the cadmium-responsive element is located in the *HinfI/HinfI* fragment of 44 bp (356/399), carried by pHOL25 (Fig. 5).

To explore the involvement of a putative AP-1 binding site, located just downstream of the *HinfI/HinfI* fragment (356/399), in cadmium-mediated induction, we repeated similar assays using THP-1 cells in which the heme oxygenase gene is transcriptionally activated by TPA (29) (Fig. 5B). Relative luciferase activities were similarly induced by cadmium in the THP-1 cells transfected with pHOL23 or pHOL25, containing this *HinfI/HinfI* fragment (356/399). In contrast, no cadmium-mediated increases were detected in the cells transfected with pHOL26 containing a putative AP-1 binding site but lacking the *HinfI/HinfI* fragment. These results are consistent with the results of HeLa cells and other cell lines. Thus, we postulate that the AP-1 site is not directly involved in the cadmium-mediated activation of the human heme oxygenase gene. It is noteworthy that TPA treatment increased the relative luciferase activities in the cells expressing any constructs used in Fig. 5B (data not shown), which is consistent with our report that TPA-responsive element is located at positions -156/-147 (29).

To localize further the cadmium-responsive element within the 44-bp *HinfI/HinfI* fragment, four synthetic oligonucleotides, Cd1, Cd2, Cd4, and Cd5, were designed (Fig. 6A) and inserted into the *SalI* site of pHOL5. It should be noted that the Cd2 oligonucleotide completely lacks the TGAGTCA motif and the Cd5 oligonucleotide contains only first 2 residues of this motif. Cadmium treatment increased the relative luciferase activity by 2–4-fold in the cells transfected with pHOLCd2 or pHOLCd5 but did not significantly increase the relative luciferase activity in the cells transfected with pHOLCd1 or pHOLCd4. These findings indicate that the cadmium-responsive element is located in the 20-bp region represented by the Cd2 oligonucleotide. Interestingly, Cd2 and Cd5 oligonucleotides contain one and two copies of a 9-bp element, GATTTTGCT, respectively, the sequence of which is also present in the mouse heme oxygenase gene (see Fig. 5A).

Presence of a Protein(s) That Binds to the Cadmium-responsive Element—To look for a protein that binds to the cadmium-responsive element, we carried out gel mobility shift assays using the ³²P-labeled Cd5 oligonucleotide of 25 bp (Fig. 6B). Two retarded bands were detected, indicating that nuclear proteins indeed bind to the Cd5 oligonucleotide. These binding activities were unchanged by the treatment with cadmium (*lanes 2 and 3*). In addition, the formation of these protein-DNA complexes was competed for by a 100-fold molar excess of the Cd2 or Cd5 oligonucleotide, but not competed for by a 100-fold molar excess of the Cd1 or Cd4 oligonucleotide (Fig. 6B). These results indicate that the proteins specifically recognize the sequence of the Cd2 oligonucleotide. Furthermore, the formation of the protein-DNA complexes was not competed for by the AP-1 oligonucleotide (*lane 8*).

Enhancer Function of the Cadmium-responsive Element—To examine whether the cadmium-responsive element has an enhancer function, we constructed another series of fusion genes with a homologous or a heterologous promoter using the 25-bp Cd5 oligonucleotide as a putative enhancer element (Fig. 7). The presence of the Cd5 oligonucleotide in both orientations with the heme oxygenase gene promoter conferred the cadmi-

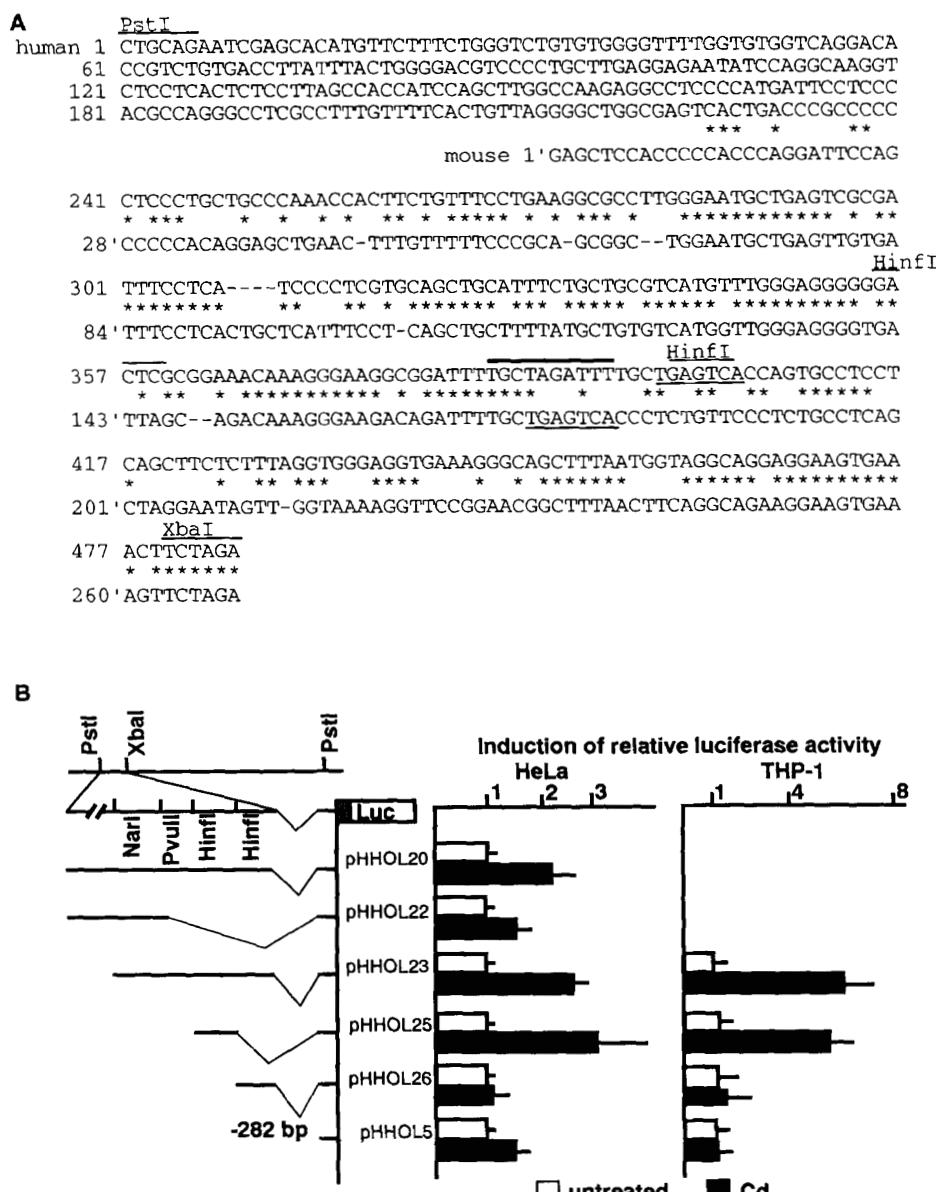


FIG. 5. Localization of the DNA segment containing the cadmium-responsive element of the human heme oxygenase gene. Panel A, the nucleotide sequence of the DNA segment containing a cadmium-responsive element. The top line represents the nucleotide sequence of the *PstI/Xba*I segment (about -4.5 kb/-4 kb) of the human heme oxygenase gene and the bottom line represents that of the DNA segment about 4 kb upstream from the transcriptional initiation site of the mouse heme oxygenase gene (40). The nucleotide sequence of the human heme oxygenase gene segment was determined from both strands, and the nucleotide residues are tentatively numbered from the *PstI* site. The nucleotide residues of the mouse gene are numbered according to the report of Alam and Zhining (40). The restriction enzymes shown were used for construction of the fusion genes. A putative core sequence of the cadmium-responsive element is marked with a bold overline and a TGAGTCA motif is underlined. Panel B, localization of a cadmium-responsive element. The *PstI/Xba*I fragment or its dissected fragments were inserted upstream of pHHOL5 (282 bp from the transcription initiation site). Thus, each fusion gene contains the deletion of about 4 kb as shown to the left. HeLa cells and THP-1 cells transfected with each fusion gene were treated for 5 h with 5 and 50 μ M cadmium (10% fetal calf serum), respectively. Other experimental procedures were the same as described in the legend to Fig. 3B. The relative luciferase activity is shown to the right.

um-mediated induction of luciferase activity. Similarly, the relative luciferase activities were induced by about 2.5-fold in the cells transfected with pSVLCd5 or pSVLCd5i containing the cadmium-responsive element in the sense or antisense orientation upstream from SV40 promoter (Fig. 7). This establishes that the cadmium-responsive element has an enhancer function.

Identification of the Cadmium-responsive Element—To identify the core sequence required for the cadmium-mediated activation, we introduced various base changes into the Cd2 oligonucleotide (375/394) and analyzed the function of each mutant element (Fig. 8A). MT1 contains the CC dinucleotides instead of the TT dinucleotides in the center of the Cd2 oligo-

nucleotide (383/384), and MT3 contains three base changes near the 5' end of the Cd2 oligonucleotide (377/379). MT5 or MT6 contains one base change of the TA dinucleotides (388/389). The mutant elements, MT1, MT3, MT5, and MT6, were able to confer the cadmium-mediated inducibility on the fusion genes, pHOLCd2/MT1, MT3, MT5, and MT6, respectively, indicating that these base changes are not directly involved in the cadmium-mediated inducibility (Fig. 8B). In contrast, MT2 containing two base changes (GC to CG) near the center of the Cd2 oligonucleotide (386/387) reduced the induction of luciferase activity, and MT4 containing two base changes (AG to GA) near the 3' end of the Cd2 oligonucleotide (389/390) were not able to lead to the cadmium-mediated induction of luciferase

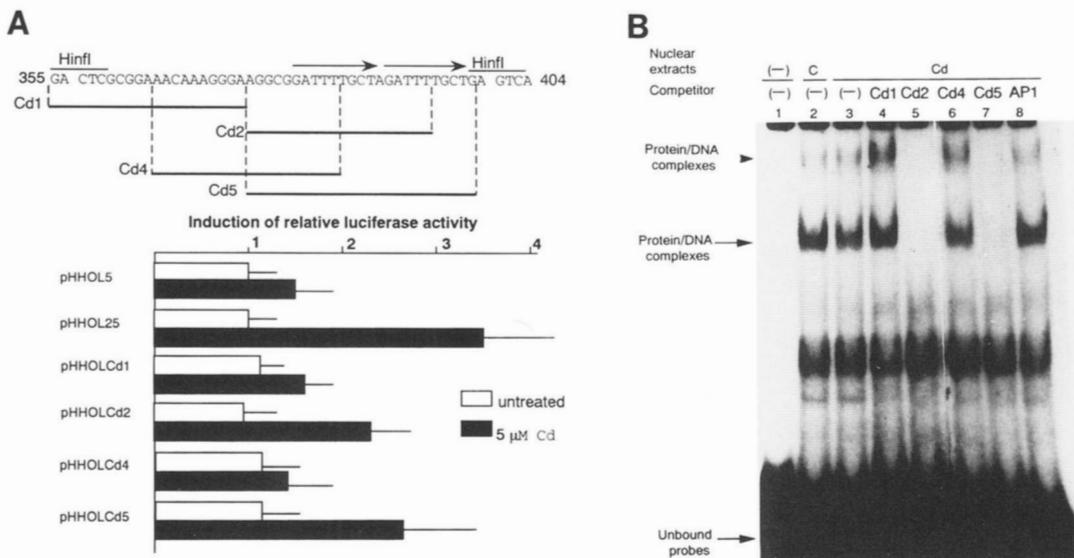


FIG. 6. Identification of the cadmium-responsive element using synthetic oligonucleotides. *Panel A*, the nucleotide sequence shown at the top is the *Hinfl/Hinfl* fragment of 44 bp containing the cadmium-responsive element localized in Fig. 5. The synthetic oligonucleotides were designed as Cd1, Cd2, Cd4, and Cd5, each region of which is represented by the thick bar. A direct repeat of 9 bp is indicated by overarrows. A bottom panel shows functional analysis of the fusion plasmids, pHOLCd1, 2, 4, and 5, containing each double-stranded oligonucleotide in the upstream of pHOL5. Other conditions were same as described in Fig. 3B. *Panel B*, identification of a protein that binds to the cadmium-responsive element. The probe used was the synthetic oligonucleotide Cd5, end-labeled with [γ -³²P]ATP. Gel mobility shift assay was carried out using no nuclear extracts (lane 1), nuclear extracts of untreated HeLa cells (lane 2), and nuclear extracts of cadmium-treated cells (lanes 3–8). Each double-stranded oligonucleotide, Cd1, Cd2, Cd4, or Cd5, was added in the reaction buffer (lanes 4–7). AP-1 consensus oligonucleotide was also added in the reaction buffer (lane 8). Major and minor protein-DNA complexes are indicated by an arrow and an arrowhead, respectively. Unbound probes are also indicated by an arrow. Other methods are described under “Experimental Procedures.”

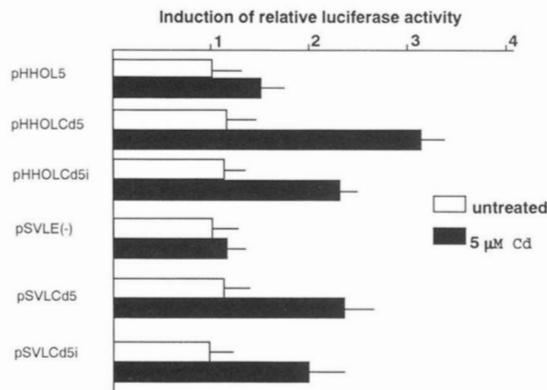


FIG. 7. Functional analysis of the cadmium-responsive element using a heterologous promoter. HeLa cells were transiently transfected with pHOL5, pHOLCd5, pHOLCd5i, pSVLE(-), pSVLCd5, or pSVLCd5i, and the expressed luciferase activity was determined. pSVL constructs contain the heterologous SV40 promoter. pHOLCd5 and pSVLCd5i carry the Cd5 oligonucleotide in the opposite direction.

activity (Fig. 8B). It is noteworthy that a base change (A to T) at position 389, represented by MT5, had no apparent effect on the cadmium-mediated inducibility. These results suggest that the GC dinucleotides (386/387) and the G residue at position 390 are essential for cadmium-mediated inducibility. Then, we performed gel mobility shift assays using the ³²P-labeled Cd2 oligonucleotide as a probe and each mutant element as competitor (Fig. 8C). Again, two protein-DNA complexes were detected as seen in Fig. 6B but with the different resolution, the pattern of which was reproducible and possibly related to the smaller size of the Cd2 oligonucleotide (20 bp). The upper bands were always faint (indicated by an arrowhead). Consistent with the functional analysis, MT2 and MT4 exerted no apparent effects on the binding of proteins to the Cd2 oligonucleotide, indicating that the proteins are unable to bind to the MT2 or MT4 element. Conversely, MT1, MT3, MT5, or MT6 completely

inhibited the formation of protein-DNA complexes, indicating that the proteins do bind to these elements. The binding of these nuclear proteins therefore correlated completely with the cadmium-mediated inducibility of the heme oxygenase gene promoter.

Effects of Other Inducers on the Cadmium-responsive Element—In addition to hemin, heme oxygenase is induced in HeLa cells by various reagents, such as sodium arsenite (39) and cobalt protoporphyrin (data not shown). To explore whether the mechanism of induction of heme oxygenase by other inducers is same as that for cadmium, we examined the effects of these inducers on the expression of luciferase activity in the cells transfected with pHOL25 containing the cadmium-responsive element. Cadmium treatment induced the expression of pHOL25, but the treatment with sodium arsenite, cobalt protoporphyrin, or hemin did not induce its expression (Fig. 9).

DISCUSSION

Heme oxygenase mRNA was increased more than 10-fold by the treatment with cadmium (Fig. 1B), although the magnitude of induction of luciferase activity by cadmium was only 2–3-fold (Fig. 3). Such a difference may simply represent that the fusion genes lack the additional element required for the maximum induction by cadmium. Alternatively, this may be due to a limitation of transient expression assays; namely, integration of the fusion genes into the genomic DNA is required for the maximum cadmium-mediated activation. It is also possible that cadmium may affect the stability of heme oxygenase mRNA (16), although cadmium was shown to act at the transcriptional level to induce heme oxygenase gene expression (Fig. 2).

Two overlapping oligonucleotides, Cd2 and Cd5, are sufficient to confer the inducibility of the fusion genes by cadmium (Fig. 6A) and were bound by nuclear proteins (Figs. 6B and 8C). Interestingly, the Cd2 oligonucleotide (375/394) contains one copy of the sequence GATTGGCT (380/388), whereas the

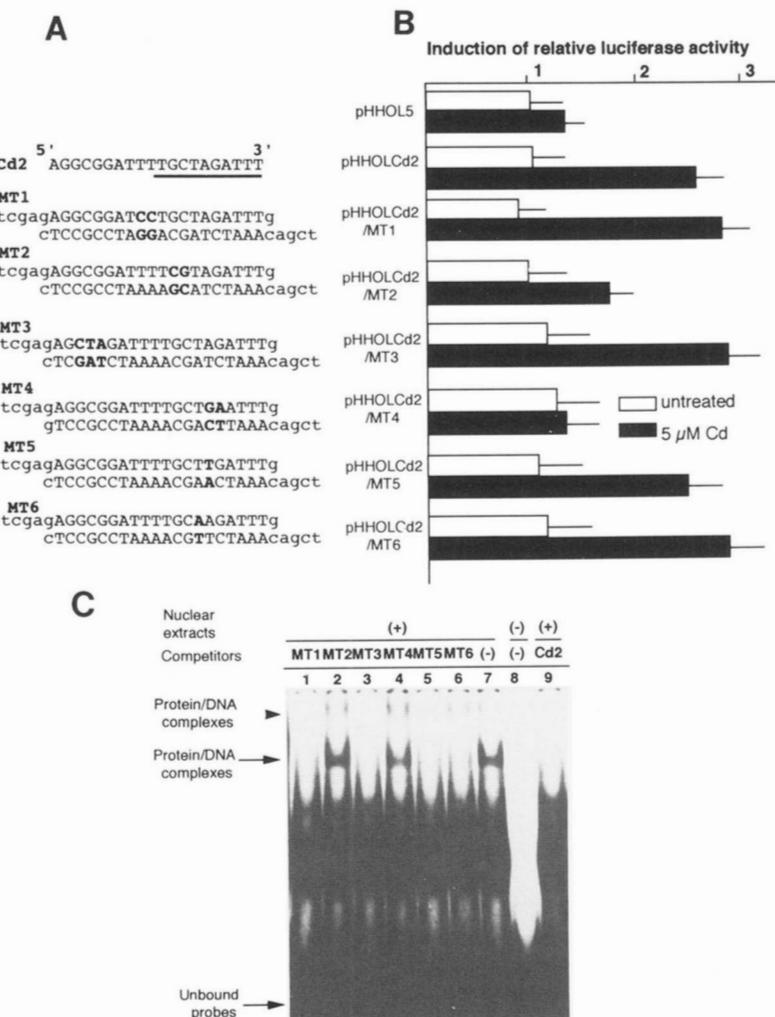


Fig. 8. Identification of the core sequence for the cadmium-responsive element. Panel A, mutant elements of Cd2 oligonucleotide. The cadmium-responsive element of 10 bp is marked with a bold underline. Six mutant elements, MT1, 2, 3, 4, 5, and 6, were synthesized by introducing base changes into Cd2 oligonucleotide. The lowercase letters represent the artificial sequence used for construction of the fusion genes, pHOLCd2 and pHOLCd2/MT1, MT2, MT3, MT4, MT5, and MT6. The effects of these base changes were evaluated by transient expression assays (panel B) and by gel mobility shift assays (panel C). The DNA probe was the ^{32}P end-labeled Cd2 oligonucleotide and the major protein-DNA complexes are indicated by an arrow.

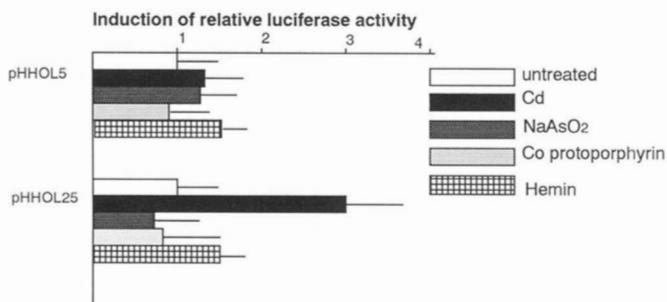


Fig. 9. Effects of other inducers on the transient expression of a fusion gene containing the cadmium-responsive element. HeLa cells, transfected with pHOL5 or pHOL25 containing the cadmium-responsive element, were treated with 5 μM cadmium, sodium arsenite, cobalt protoporphyrin, or hemin. Other experimental procedures were the same as described in Fig. 3B.

Cd5 oligonucleotide (375/399) contains an additional copy of the same sequence (390/398). However, the magnitude of the activation is not different between pHOLCd2 and pHOLCd5, indicating that this 9-bp element alone is not sufficient for cadmium inducibility and that the cadmium-responsive element is located within the 20-bp element, represented by the Cd2 oligonucleotide (Fig. 6A). Incidentally, one copy of the se-

quence identical to the 3' half of the Cd5 oligonucleotide, AGATTTTGCTG (389/399), is also present at similar position in the mouse heme oxygenase gene (40). However, Alam and Zhining reported that transient expression of the reporter gene carrying this mouse element was not increased by cadmium (40). It is also evident that the TGCT sequence (395/399) at the 3' half of the above 11-bp element is not required for cadmium-mediated induction of the human heme oxygenase gene because Cd2 oligonucleotide lacks this sequence (see Fig. 6A). It should be noted that the sequences flanking at the 5' side of this 11-bp element are different between human and mouse heme oxygenase genes (see Fig. 5A); namely, the human heme oxygenase gene contains the TGCT sequence (385/388) but the mouse gene contains the AGAC sequence. Thus, this flanking TGCT sequence may be crucial for the cadmium-mediated induction of the human heme oxygenase gene. Indeed, functional analysis of the Cd2 mutant elements established that the GC dinucleotides (386/387) are essential for cadmium-mediated inducibility and protein binding, but the two base changes near the 5' end and in the center of the Cd2 element, GCG (377/379) to CTA and TT (383/384) to CC, were without effect (see Fig. 8). In addition, the G residue at position 390 is required for cadmium inducibility and protein binding. These results indicate that the sequence crucial for cadmium inducibility exists in the

3' half of the Cd2 element. Considering all these facts, we suggest that the sequence TGCTAGATT (385/394) is responsible for cadmium-mediated induction of the human heme oxygenase gene (see Figs. 5A and 8A).

We identified the two binding activities in HeLa cells which specifically recognize the cadmium-responsive element, although these DNA binding activities were not altered by the treatment with cadmium. Because of a strong correlation between cadmium-mediated inducibility and *in vitro* protein binding (Fig. 8), we propose that the protein(s) detected in this study is involved in the cadmium-mediated induction of the human heme oxygenase gene. The two protein-DNA complexes may represent the two different DNA-binding proteins or a common DNA-binding protein with different interacting protein(s). In either case, we assume that cadmium treatment may increase the *trans*-activation activity but not the DNA binding activity of the detected protein(s). Such an example is known in AP-2 that is involved in the induction of human metallothionein II_A gene expression mediated by TPA; namely, the DNA binding activity of AP-2 is not affected by TPA, but its transcriptional stimulatory activity is enhanced (41). More recently, we have identified the *cis*-regulatory element and *trans*-acting factors that are responsible for TPA-mediated induction of human heme oxygenase in myelomonocytic cell lines (29). In this case, the DNA binding activities were unchanged by TPA. Alternatively, cadmium treatment may alter the property of another factor that interacts with the protein(s) binding to the cadmium-responsive element, which in turn leads to transcriptional activation of the heme oxygenase gene. Purification of the binding protein(s) and its cDNA cloning are required to address these questions.

A sequence TGAGTCA, identical to the TPA-responsive element, is located downstream from the 3' end of the cadmium-responsive element (Fig. 5A). However, heme oxygenase is not induced by TPA in human cell lines, such as HeLa cells, MeWo melanoma cells, and MRC-5 diploid fibroblasts (29), but was induced by TPA in a FEK₄ human skin fibroblast cell line (48) and in three human myelomonocytic cell lines, including THP-1 (29). It was also reported that the mouse heme oxygenase gene is activated by exposure to TPA in BALB/c 3T3 (49) and M1 cells (50). Furthermore, Alam and Zhining demonstrated that AP-1 mediates the induction of the mouse heme oxygenase gene by TPA (40). Interestingly, the AP-1 binding site is located about 4 kb upstream from the transcription initiation site of the mouse heme oxygenase gene (40), which is similar to the position of a TGAGTCA motif found in the human heme oxygenase gene (Fig. 5A). Thus, we also analyzed the function of a putative AP-1 binding site using THP-1 cells (Fig. 5B), confirming that this element is not directly involved in the cadmium-mediated activation. Cadmium treatment increased the expression of pHOLCd2 lacking the core sequence of AP-1 binding site, TGAGTCA (Fig. 6), and synthetic AP-1 oligonucleotide did not inhibit the binding of nuclear protein to the 25-bp Cd5 oligonucleotide (Fig. 6) and the 20-bp Cd2 oligonucleotide (data not shown). We thus conclude that the TGAGTCA motif is not directly involved in the cadmium-mediated induction of the human heme oxygenase gene.

A mammalian metal-responsive element, TGCRCNC (18, 42, 43), from the metallothionein gene promoter, is not found in the human heme oxygenase gene promoter region so far analyzed. Conversely, a putative core sequence of the cadmium-responsive element, TGCTAGATT, is not present in the promoter region of the human metallothionein gene (18, 44, 45). The cadmium-responsive element differs from the metal-responsive element in all except the first 3 bases. Incidentally, the TTTGC sequence, overlapping with the 5' end of the cadmium-respon-

sive element (see Fig. 5A), is identical to the sequence overlapping with the metal-responsive elements of human metallothionein I_A and II_A genes and mouse metallothionein I and II_A genes (42). However, it should be noted that the TT dinucleotides at its 5' end are not crucial for cadmium inducibility and *in vitro* protein binding (see Fig. 8). Recently, two transcription factors, MTF-1 (46) and MBF-I (47), were identified and shown to bind to the metal-responsive element of the mouse metallothionein I gene and activate its transcription. The DNA binding activity of MTF-1 was induced by the treatment with zinc (46), whereas that of MBF-I was not (47). It remains to be investigated whether these factors are also involved in the cadmium-mediated induction of the heme oxygenase gene. However, we assume that the mechanism of the metal regulation of the human heme oxygenase gene may be different from that of the metallothionein gene because the core sequence of the cadmium-responsive element is distinct from the metal-responsive element, and zinc did not increase heme oxygenase gene expression in HeLa cells (data not shown).

We have provided evidence that the mechanism of cadmium-mediated induction of human heme oxygenase is different from that mediated by hemin, sodium arsenite, or cobalt protoporphyrin, since these inducers did not increase the luciferase activity expressed in the cells transfected with pHOL25 carrying the cadmium-responsive element (see Fig. 9). These results may reflect the difference in the biological implication of the induction by each agent. It is conceivable that the induction of heme oxygenase by either hemin (iron protoporphyrin) or possibly cobalt protoporphyrin is a beneficial response that allows the organism to maintain the levels of cellular heme, a potential prooxidant, whereas the induction by sodium arsenite may contribute to the protection against oxidant stress by increasing the levels of bilirubin, an antioxidant (4, 14).

The physiological relevance of the cadmium-mediated induction of heme oxygenase is not known. It is reasonable to assume that this induction may reflect the protection against metal toxicity, the protection mechanism of which, however, may be different from that of metallothionein as described in Introduction. Alternatively, the cadmium-mediated induction may reflect the replacement with cadmium of a physiological mediator for a signal transduction system, leading to transcriptional activation of the human heme oxygenase gene. Further characterization of the 10-bp sequence and its binding protein(s) may help us understand biological implication of the cadmium-mediated induction of heme oxygenase.

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