Participation of altered upstream stimulatory factor in the induction of rat heme oxygenase-1 by cadmium

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ABSTRACT
We have reported that an upstream stimulatory factor (USF) binding site is functional in transcription of the heme oxygenase-1 gene. In this study, we examined the role of USF in the induced state. By transient expression analyses with the chloramphenicol acetyltransferase gene, we found that the USF binding site plays an important role in the induction of rat heme oxygenase-1 by cadmium, but not by hemin. To elucidate the role of USF, we prepared USF-rich nuclear extracts from control and cadmium-treated rat liver. On electrophoretic mobility shift assay using control nuclear proteins, one slowly migrating band was detected, whereas using nuclear proteins of cadmium-treated rat liver, two fast migrating bands were detected. The molecular masses of the two subunits of USF prepared from cadmium-treated rat liver were ∼34 kDa as determined by UV cross-linking and subsequent SDS–PAGE, while the two subunits of native USF were 43 kDa and 44 kDa. DNase I footprinting analysis revealed that both the nuclear proteins bound to the same region including the USF binding site. We therefore suppose that cadmium causes some structural changes in the two proteins of USF and that the altered USF participates in the effective initiation of transcription of the rat heme oxygenase-1 gene.

INTRODUCTION
Microsomal heme oxygenase catalyzes the oxidative degradation of heme to biliverdin, carbon monoxide and iron, in cooperation with NADPH cytochrome P-450 reductase, which functions as an electron donor, and biliverdin is subsequently reduced to bilirubin by biliverdin reductase in the soluble cell fraction (1). Heme oxygenase has two isozymes, an inducible enzyme referred to as heme oxygenase-1 and a constitutive enzyme named heme oxygenase-2 (2). Heme oxygenase-1, a 33 kDa protein (3,4), is mainly distributed in reticuloendothelial cell-rich tissues such as spleen and liver (1). In 1970, Tenhunen et al. (5) found that the activity of rat liver heme oxygenase-1 was increased by injection with hemin or hemoglobin. This is unique, because substrate-mediated induction in animals is quite rare. Since then there have been many reports describing how a number of non-heme substances, such as heavy metals (6), some organic compounds (7,8), endotoxins (9), interleukins (10–12), UV light (13) and heat shock (14), significantly induce heme oxygenase-1. The physiological significance of the inducibility of heme oxygenase-1 has remained obscure. However, the most likely explanation is that heme oxygenase-1 works as a defense mechanism against oxidative stress, because biliverdin and bilirubin may function as potent scavengers of oxygen radicals (15). In addition, carbon monoxide is now suggested to have a function in neurotransmission and vascular tone regulation (16–18).

Previously, Sato et al. (19,20) reported that binding of the upstream stimulatory factor (USF) consisting of 44 kDa and 43 kDa proteins (21–23) to a USF binding site of the heme oxygenase-1 gene, located at −51 to −35 bp from the transcription initiation site, was essential for basal expression of this enzyme; the USF binding site was first found in the adenovirus-2 major late promoter gene (24–26), its central motif being CACGTG (24). Among the above-described inducers, we have paid especial attention to hemin and cadmium, since hemin is a substrate and cadmium is one of the most potent inducers (27). So, in this study, we intended to clarify the role of USF in the induced state and found that USF participated in the induction of heme oxygenase-1 by cadmium but not by hemin. Moreover, we found that cadmium caused an alteration in USF. The present paper is concerned with an investigation of these problems.

MATERIALS AND METHODS
Materials
The following compounds were used: restriction endonucleases and T4 polynucleotide kinase from Toyobo; DNase I from Boehringer; [γ-32P]ATP, [α-32P]dCTP and chloramphenicol D-threo-[dichloroacetyl-1,2-14C] from New England Nuclear; heparin–agarose from Sigma; poly(dI-dC)·poly(dI-dC), Sephadex G-25 and a molecular mass determination kit from Pharmacia. Rabbit polyclonal antibodies raised against a synthetic peptide corresponding to amino acids 291–310 of the 43 kDa protein of human USF were from Santa Cruz Biotechnology. These antibodies react with both the 43 kDa and 44 kDa proteins of humans, mice and rats. Non-specific rabbit IgG was from Jackson Immuno Research Laboratories. The synthetic oligonucleotides

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5′-CGGCCACACGTGACTCGAG-3′ and 5′-CTCGAGTCA-CGTTGTTGCCC-3′, which contain the USF binding site, were products of Sawady Technology and were used as synthetic 20 bp DNA after being annealed. DNA fragments of the rat heme oxygenase-1 gene containing the USF binding site were prepared from plasmid SpRHO8 (19). For labeling the 5′-end of DNA fragments, [γ-32P]ATP and T4 polynucleotide kinase were used.

Plasmid constructions

The SV40 polyadenylation sequence of pSV2cat (28) excised with HindIII and BamHI was ligated into Smal and BamHI sites of pUC19, because the presence of a polyadenylation site just before the promoter region of the chloramphenical acetyltransferase (CAT) gene was reported to decrease non-specific expression of CAT (29). Thus the obtained plasmid was cut with HindIII and PvuII. The gap between the HindIII and PvuII sites was filled in with a HindIII–BamHI (blunt) fragment of pSV2cat, which contains the CAT gene, polyadenylation site and small t intron from SV40. This plasmid, carrying no promoter sequence, was named pUC00cat and was used as a negative control for the CAT assay. pUC00cat was cut with SrfI, both ends were filled in and lastly it was digested with HindIII. The SV40 promoter region of pSV2cat cut out with PvuII and HindIII and was ligated into SrfI (blunt) and HindIII sites of pUC00cat. This plasmid, carrying the whole sequence of the SV40 promoter, was referred to as pUCcat and was used as a positive control for the CAT assay. The expression plasmids including the 5′ upstream region of the rat heme oxygenase-1 gene were constructed as follows. SpRHO8 (19), which contains the −748 to +2116 fragment of rat heme oxygenase-1, was linearized at the BamHI site and the deletion construct was generated with exonuclease III and mung bean nuclease. Dideoxy sequencing showed that the deletion mutant contained the region between −748 and +53. The deletion mutant was digested with NdeI and the NdeI-deletion fragment (−137 to +53) was filled in at both ends. The fragment was cloned into HindIII (blunt) and BamHI (blunt) sites of pUC00cat to yield pUCNcat. In the same way, a XhoI-deletion fragment (−40 to +53) was inserted into the same sites of pUC00cat to yield pUCXcat. A Eco72I–XhoI fragment (−44 to −40) was dissected from pUCXcat, filled in and self-ligated to generate pUCEcat.

Cell culture, transfection and CAT assay

Rat C6 glioma cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum under humidified air containing 5% CO2 at 37°C. Glioma cells (1–2 × 10⁶ per 9 cm plate) were transiently transfected with plasmids by the calcium phosphate method (30) with slight modification. The DNA mixture was composed of 15 μg expression plasmid and 5 μg β-galactosidase expression vector pCH110 (Pharmacia). After 6 h the cells were treated with 15% glycerol for 1 min, cultured in serum-containing medium for 40 h and then incubated in serum-free medium containing the respective inducers of heme oxygenase-1 except for hydrogen peroxide. Three hours later, the cells were washed twice with phosphate-buffered saline and cultured in serum-containing medium for 4 h. Hydrogen peroxide treatment was carried out in serum-containing medium for 1 h, with a recovery period of 6 h. The concentrations of inducers used were: CdCl2, 100 μM; CoCl2, 500 μM; hemin, 20 μM; hydrogen peroxide, 600 μM. Cell extracts prepared by three cycles of freezing and thawing followed by centrifugation were assayed for CAT (30) and β-galactosidase (30) activities. The radioactivity of acetylated chloramphenicol on thin layer plates was quantified with an imaging analyzer BAS 2000 (Fuji Film). The CAT activity was normalized to β-galactosidase activity (30).

Northern blot analysis

Rat glioma cells were cultivated in serum-free medium containing the respective inducers of heme oxygenase-1 except for hydrogen peroxide. After 1 h, the cells were washed twice with phosphate-buffered saline, followed by cultivation in serum-containing medium for 3 h. Hydrogen peroxide treatment was carried out in serum-containing medium for 1 h, with a recovery period of 3 h. Total RNA, prepared by the method of Chirgwin et al. (31), was separated on a denaturing formaldehyde–agarose (1%) gel, transferred to a nylon membrane (Magnagraph), fixed to the membrane and hybridized with 32P-labeled probes. The probe was used an EcoRI–HindIII fragment (+88 to +970) of rat heme oxygenase-1 cDNA (32) labeled with [α-32P]dCTP by the random priming method (33). The same filter was also subjected to rehybridization with a 32P-labeled rat glyceraldehyde-3-phosphate dehydrogenase cDNA probe (34). The filter was autoradiographed with X-ray film. The radioactivity was determined using the imaging analyzer and an ATTO Densitograph (ATTO).

Preparation of a USF-rich fraction from nuclear extract

Wistar rats (~250–300 g) were treated i.p. with saline with or without CdCl2 (14 mg/kg body wt) (27). Two hours later they were killed by decapitation and their liver was excised. Blood was washed out with cold saline injected through the portal vein. Crude nuclear extracts prepared by the method of Dignam et al. (35) were treated with an equal volume of saturated ammonium sulfate solution at pH 7.0 and stood on ice overnight. The precipitates collected by centrifugation were dissolved in 20 mM HEPES–KOH buffer, pH 7.9, containing 0.1 M KCl, 0.2 mM EDTA, 1 mM dithiothreitol and 15% glycerol and dialyzed against the same buffer. The clear supernatant obtained by centrifugation was applied to a Sephadex G-25 column equilibrated with TM buffer (50 mM Tris–HCl, pH 7.9, 12.5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol and 20% glycerol) containing 0.1 M KCl (19). The fraction containing proteins was then applied to a heparin–agarose column equilibrated with the same buffer as that used for Sephadex G-25 chromatography. The column was washed with TM buffer containing 0.3 M KCl and then proteins bound to the heparin–agarose were eluted with TM buffer containing 0.6 M KCl and the resulting eluate was used as the USF fraction.

Electrophoretic mobility shift assay

Reaction mixture containing 20 mM HEPES–KOH, pH 7.9, 40 mM KCl, 4% Ficoll, 6 mM MgCl2, labeled DNA fragments (10 fmol, 10 000 c.p.m.), 1.5 μg poly(dI-dC)-poly(dI-dC) and the USF fraction (5 μg protein) in a final volume of 20 μl was incubated for 20 min at 25°C. Then the mixture was loaded onto a 4%
polyacrylamide gel containing 50 mM Tris and 380 mM glycine (36) and subjected to electrophoresis for 60–90 min at 10 V/cm.

**DNase I footprinting analysis**

The *Nde*I–*Nco*I DNA fragment, end-labeled at the *Nde*I site (0–50 µg protein) in 30 µl 20 mM HEPES–KOH, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 2 mM MgCl₂, 5% glycerol and 5 µg poly(dI-dC)·poly(dI-dC) for 20 min at 25°C and partially digested with 5 µl DNase I (0.04 U in 20 mM MgCl₂ and 10 mM CaCl₂) for 1 min at 25°C. The digestion was stopped by addition of 75 µl 12.5 mM EDTA and the mixture was extracted with phenol/CHCl₃. Samples were then separated in an 8% polyacrylamide–8 M urea sequencing gel (37).

**UV cross-linking of DNA–protein complexes**

USF factions (10 µg protein each) were incubated with the labeled synthetic 20 bp DNA (10–20 fmol, 30 000 c.p.m.) in 30 µl 20 mM HEPES–KOH, pH 7.9, 40 mM KCl, 4% Ficoll, 6 mM MgCl₂ and 5 µg poly(dI-dC)·poly(dI-dC) for 20 min at 25°C and irradiated with a UV lamp (maximum emission wavelength 254 nm, maximum intensity 730 µW/cm² at a distance of 15 cm from the UV source) for 30 min (38,39). Then cross-linked samples were subjected to electrophoresis in a 12% SDS–polyacrylamide gel (40).

**RESULTS**

**Participation of USF in the induction of rat heme oxygenase-1 by cadmium**

Sato et al. (19) found that the USF binding site is functional in basal transcription of the heme oxygenase-1 gene in rat glioma cells. Therefore, we used rat glioma cells for the present study. First, we tested whether these cells respond to well-known inducers of heme oxygenase-1 such as cadmium, cobalt, hemin and hydrogen peroxide. Figure 1 shows that these inducers could stimulate transcription of the heme oxygenase-1 gene, but not glyceraldehyde-3-phosphate dehydrogenase, a housekeeping gene. These observations indicate the validity of the use of rat glioma cells for transient expression analysis of the promoter region of the heme oxygenase-1 gene.

Next we studied whether the USF binding site was functional or not in transcribing the heme oxygenase-1 gene in the induced state. Glioma cells transfected with pUCNcat were treated with the respective inducers and then CAT activity was assayed. This plasmid is composed of 137 nt of 5′ flanking region, including the USF binding site, 53 nt of exon 1 of the heme oxygenase-1 gene and the whole coding region of CAT protein (Fig. 2A). As shown in Figure 2B, only cadmium could increase CAT activity, ~4.5-fold. These findings suggest that the USF binding site is functional in the cadmium-induced increase in CAT activity. To confirm this possibility, we constructed two plasmids, pUCEcat, which lacks a GTGAC sequence within the USF binding site, and pUCCXcat, which lacks the 5′ flanking region, including the USF binding site, from nucleotide C at position −40 (Fig. 3). As shown in Figure 3, cadmium failed to increase CAT activity in cells
transfected with these plasmids, indicating that the USF binding site plays a critical role in induction of heme oxygenase-1 by cadmium. Furthermore, in agreement with the previous finding that the USF binding site was essential for basal expression of heme oxygenase-1 (19,20), basal CAT activity of cells transfected with pUCXcat was extremely low.

Presence of altered USF retaining binding activity in nuclei of cadmium-treated rat liver

Recently, Nascimento et al. (41) reported that UV A irradiation, an inducer of heme oxygenase-1, caused modification of USF. Therefore, we next investigated if cadmium caused a similar modification or not. The USF fraction prepared from control rat liver was mixed with the end-labeled 49 bp PvuII–PvuII (−59 to −11) fragment containing the USF binding site and subjected to an electrophoretic mobility shift assay. As shown in Figure 4, a distinct band attributable to a DNA–protein complex was detected (lane 4) and on addition of a 500-fold molar excess of synthetic 20 bp DNA containing the core sequence (CACGAG) of the USF binding site, the DNA–protein complex band disappeared completely (lane 5). These observations indicate that the USF in control nuclei bound to the USF binding site of the rat heme oxygenase-1 gene, consistent with the previous finding (19). The bands near the bottom of the gel were due to unbound DNA fragments, because they were also observed on electrophoresis of labeled 49 bp DNA alone (lane 1). In contrast, two distinct, faster migrating bands were observed after incubating the DNA fragment with the USF fraction from cadmium-treated rat liver (lane 2). Interestingly, neither band was seen in the presence of a 500-fold excess of unlabeled synthetic 20 bp DNA (lane 3). In a similar experiment in which a control USF fraction treated exogenously with cadmium was used, the two fast migrating bands did not appear and only one band corresponding to the band in lane 4 was detected (data not shown). This suggests that cadmium does not affect the USF directly.

To determine whether the binding proteins in the USF fractions of untreated and cadmium-treated rat liver were identical to USF, we treated the DNA–protein complexes with rabbit IgG containing antibodies against USF and then subjected the mixtures to an electrophoretic mobility shift assay (Fig. 5). On treatment with anti-USF antibodies (lane 6) the band due to the complex of DNA and protein(s) in the control USF fraction (lane 5) was considerably decreased, but was not affected by treatment with non-specific rabbit IgG (lane 7). Moreover, the two bands of the complexes of DNA and protein obtained from cadmium-treated rat liver nuclei (lane 2) were no longer detectable after treatment with anti-USF antibodies (lane 3). In electrophoretic mobility shift assays with specific antibodies, two patterns have been reported; one a supershift and the other competition, though the latter case is not so frequent (42–44). However, it is noteworthy that Paterson et al. (44), who employed the same antibodies in their study, also observed not supershift but competition. At any rate, our findings indicate that the binding proteins in cadmium-treated nuclei are altered forms of USF.

To identify the binding region more precisely, we analyzed the NdeI–NcoI (−137 to +260) fragment containing the above 49 bp fragment by DNase I footprinting analysis. Figure 6 shows that proteins in both the USF fractions protect the same region (−56 to −37) of the rat heme oxygenase-1 gene from digestion. The
Figure 5. Electrophoretic mobility shift assay carried out with specific antibodies to USF. Labeled synthetic 20 bp DNA was used as a probe. When necessary, 1 µg specific or non-specific IgG was added to the reaction mixture. Lane 1, probe alone; lane 2, probe and Cd(⁺⁺); lane 3, probe, Cd(⁺⁺) and specific IgG; lane 4, probe, Cd(⁺⁺) and non-specific IgG; lane 5, probe and Cd(⁻⁻); lane 6, probe, Cd(⁻⁻) and specific IgG; lane 7, probe, Cd(⁻⁻) and non-specific IgG.

Figure 6. DNase I footprinting analysis. The NdeI–NcoI fragment end-labeled at the NdeI site was incubated with various amounts of the USF fraction (0–50 µg protein as indicated at the top of the lanes). The protected region is shown on the right side of the figure. In lane (G+A), the same DNA fragment that had undergone a (G+A) reaction (37) was loaded as a size maker. UV cross-linking of the DNA–USF complexes and estimation of the molecular mass of bound USF

For determination of the molecular masses of native and altered USF, mixtures of the labeled synthetic 20 bp DNA (~12 kDa) and each USF fraction were subjected to UV irradiation and then examined by SDS–PAGE (Fig. 7A). On electrophoresis, the cross-linked sample of the DNA and the control USF fraction gave two broad bands, B-2 and B-3 (lane 3), whereas without UV irradiation no bands were seen (data not shown). The presence of a 500-fold excess of unlabeled 20 bp DNA at the time of UV irradiation completely abolished formation of the complexes of labeled DNA and USF (lane 4). The molecular masses of B-2 and B-3 were estimated to be 99 and 55 kDa respectively, by comparison of the migration distances of the centers of the broad bands with those of marker proteins. By subtracting the 12 kDa of the synthetic oligonucleotide from these observed values, we estimated that the molecular masses of the proteins in the B-2 and B-3 bands were 87 and 43 kDa respectively. The positions of marker proteins are indicated on the right. (B) Schematic interpretation of (A). Details are described in the text.

DISCUSSION

So far, the cadmium-responsive cis elements of the human and mouse heme oxygenase-1 gene have been reported by Takeda et al. (45) and Alam et al. (46–49) respectively. Takeda et al. identified the 10 bp cis-acting element of the human heme oxygenase-1 gene, located ~4 kbp upstream from the transcription initiation...
site, as responsible for cadmium-mediated induction, but not for hemin-, cobalt- or zinc-mediated induction (45). On the other hand, Alam et al. (47) reported that activation of the mouse heme oxygenase-1 gene by 12-O-tetradecanoylphorbol-13-acetate, heme and cadmium is mediated by a 268 bp fragment located ∼4 kbp upstream of the transcription initiation site (46–48). In addition, Alam et al. (49) found that a 161 bp fragment located ∼10 kbp upstream of the transcription initiation site was a second regulatory region for cadmium- and heme-mediated induction. Although the relation between these cadmium-responsive elements and USF is unknown, the present study demonstrates that the cis USF binding site plays an important role in cadmium-mediated induction of rat heme oxygenase-1.

USF, a helix–loop–helix transcription factor that binds specifically to the USF binding site, consists of 43 and 44 kDa proteins (21–23), although molecular masses of 33.5 kDa for the former (50) and 36.9 kDa for the latter (22) have been calculated from the nucleotide sequences of their genes. The present study shows that cadmium administration causes structural change in USF resulting in an increase in the mobilities of the two proteins in a SDS–polyacrylamide gel and the loss of their ability to dimerize, although the actual alteration remains unclear. However, they were still able to bind to the USF binding site, though with a lower affinity than native USF, as discussed later. The fact that only the two fast migrating bands were detected (Fig. 4) suggests that native USF was not present in cadmium-treated rat liver and that each altered protein bound to the USF binding site as a monomer, to form a DNA–altered 44 kDa protein or DNA–altered 43 kDa protein complex. We observed that treatment of both the USF fractions with calf intestinal phosphatase or λ-phosphatase did not alter their electrophoretic mobilities from those seen in Figure 4 (data not shown). This indicates that phosphorylation and dephosphorylation are not involved in the suspected structural changes. In addition, we examined whether cadmium increased the amount of USF in rat glioma cells by Northern blot analysis, but we could find no differences between cadmium-treated and untreated cells (not shown), supporting our view that this metal causes structural changes in USF.

On the other hand, Gregor et al. (50) reported the interesting finding that truncated human USF retaining the helix–loop–helix domain but lacking a part of the C-terminal leucine repeat region could bind to the USF binding site but failed to dimerize. Their report raises another possibility, that a protease that is activated by cadmium treatment may cut both the proteins at positions near the C-terminal side within the leucine repeat region in such a way that the resulting truncated peptides still react well with the antibodies. However, this possibility may be unlikely, because the antibodies used here recognize a C-terminal sequence consisting of only 20 amino acids and because the difference between the migration distances on a SDS–polyacrylamide gel of native and altered USF correspond to ∼8 kDa.

The present footprinting analysis showed that both the USF fractions protected the same sequence, whereas USF modified by UV irradiation protected only the upstream half of the region protected by native USF (41). The extent of protection by 50 µg of USF fraction prepared from cadmium-treated rat liver was similar to that by 30 µg of USF fraction of control rat liver (Fig. 6). This suggests that native USF binds more tightly than altered USF, assuming that equal amounts of USF are present in these USF fractions. This view was consistent with the finding that on treatment with specific antibodies, the band attributable to the DNA–native USF complex did not disappear completely, whereas the bands of the complex of DNA–altered USF did (Fig. 5).

When the complex of labeled DNA and native USF was subjected to UV cross-linking, two cross-linked forms appeared (Fig. 7). Dostatni et al. (51) reported that even when a complex of DNA and dimer proteins was cross-linked by UV irradiation, two types of complex, DNA–monomer and DNA–dimer, were formed. The former corresponds to linking of one protein to one side of the palindrome and the latter to cross-linking of two proteins to the symmetrical site. Thus, as illustrated in Figure 7B, we assume that bands B-2, B-3 and B-1 correspond to a complex of DNA and heterodimer, a mixture of complexes of DNA and 44 kDa protein and DNA and 43 kDa protein and a mixture of the complexes of DNA and the 34 kDa protein resulting from 44 kDa protein and DNA and the 34 kDa protein resulting from 43 kDa protein respectively. These observations, together with the results in Figure 4, suggest that native USF binds to the USF binding site as a heterodimer and support the view that USF binds to the USF binding site as a dimer (21,22,50), although there is a report that it binds to DNA as a monomer (52).

There have been some reports that USF functions in the basal expression of some eukaryotic genes, such as metallothionein I (53,54), rat γ-fibrinogen (55,56) and liver-specific rat pyruvate kinase (57,58). The present report is probably the first one describing USF working in the induced state. Nascimento et al. (41) reported a similar alteration of USF in UVA-irradiated human skin fibroblasts, but they did not demonstrate its role in expression of the heme oxygenase-1 gene. In general, loss of dimerization ability of a transcription factor and decrease in its binding ability to DNA result in a decrease in its activity. However, our studies suggest that the altered USF possessing these properties is more functional than native USF. Hence, we suppose that the altered form of USF would show greater cooperativity with RNA polymerase II and basic transcription factors necessary for the initiation of transcription of the rat heme oxygenase-1 gene.

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