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Cadmium-responsive Element of the Human Heme Oxygenase-1 Gene Mediates Heat Shock Factor 1-dependent Transcriptional Activation*

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Transcription of a number of mammalian genes is activated by heavy metals, but mechanisms of signaling and transcriptional regulation are not well understood. From a comparison of heavy metal responses of several human genes, it was noted that the heme oxygenase-1 (HO-1) gene is quite similar in the spectrum of metal response and induction kinetics to the heat shock protein 70 (HSP70) gene, suggesting a common regulatory mechanism shared by these genes. The cadmium-responsive element (CdRE) known to be responsible for the metal regulation of *ho-1* formed complexes with proteins from heavy metal-treated HeLa cells in an electrophoretic mobility shift assay (EMSA). These complexes were indistinguishable in mobility from those formed by the heat shock factor 1 (HSF1) and the heat shock element involved in *hsp70* regulation, suggesting the involvement of HSF1 also in the CdRE complexes. Competitive EMSA and supershift analysis with an anti-HSF1 antibody revealed that HSF1 was in fact a component of the CdRE complexes. A fine analysis on the affinity of HSF1 to a series of mutant CdRE sequences showed that HSF1 recognizes a sequence motif TnCTAGA. Transient transfection analysis with overexpressed recombinant HSF1 demonstrated that CdRE has HSF1-dependent enhancer-like activity that requires direct binding of HSF1. In the absence of overexpressed HSF1, however, CdRE by itself was insufficient to mediate heavy metal-induced transcription, suggesting requirement of additional regulatory sequences. The finding that HSF1 is directly involved in the regulation of *ho-1* with an anti-oxidative role revealed a new aspect of the biological defense mechanism.

It is known that heavy metals activate transcription of a number of mammalian genes including those coding for metallothionein (1), heme oxygenase-1 (2), heat shock protein 70 (3), Fos (4), and Jun (5). However, it remains unclear whether common regulatory mechanisms are shared by these genes or not. For some of them, molecular mechanisms underlying the heavy metal induction have been studied. With regard to the genes coding for metallothioneins, a family of low molecular weight heavy metal-binding proteins, metal signals are transduced by a transcription factor MTF-1[3] (MRE-binding transcription factor-1) and its target sequence MRE (metal responsive element) (6, 7). It has been reported that signaling to MTF-1 may involve several protein kinases (8, 9). The gene coding for human heme oxygenase-1 (HO-1), which is involved in heme metabolism as well as protection against oxidative stress, is activated by cadmium and other heavy metals (2, 10). It has been shown that CdRE (cadmium-responsive element) located at ~4 kb upstream from the transcription initiation site (TIS) plays an essential role in cadmium induction (2). A nucleolar protein pescadillo has recently been reported to have an ability to mediate transcription via CdRE (11). On the other hand, it has been suggested that the nuclear export of a negative regulator Bach 1 is involved in the metal response of *ho-1* (12), but its relationship with the CdRE-mediated transcriptional activation is unclear. In the heavy metal induction of the heat shock protein 70 (HSP70) gene, it has been suggested that a signal from cadmium is mediated by heat shock factor 1 (HSF1) and its recognition site heat shock element (HSE) (3, 10, 13). Metal responses mediated by HSE and MRE have been considered to be independent of each other (10, 14), but recently a cross-talk of these two pathways has been suggested (15).

In an attempt to obtain a comprehensive view in heavy metal responses of human genes, we have been studying on transcriptome modulated by heavy metals (16), and the regulatory mechanisms of several metal-inducible genes as well as their interrelationships (10, 15). In the latter study, human *hsp70-1a* (indicated as *hsp70* hereafter), *MT-2A*, and *c-fos* showed differential responses to heavy metals, suggesting no direct relationship among their mechanisms of induction (10). However, we noted that a pair of genes, namely human *ho-1* and *hsp70*, exhibited closely similar metal responses. In the present work, we studied whether these two genes share a common molecular mechanism in heavy metal-induced transcriptional activation.

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[3] The abbreviations used are: MTF-1, MRE-binding transcription factor-1; MRE, metal responsive element; HO-1, heme oxygenase-1; CdRE, cadmium-responsive element; TIS, transcription initiation site; HSP70, heat shock protein 70; HSF1, heat shock factor 1; HSE, heat shock element; WCE, whole cell extract; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; βGal, β-galactosidase; MARE, Maf recognition element.

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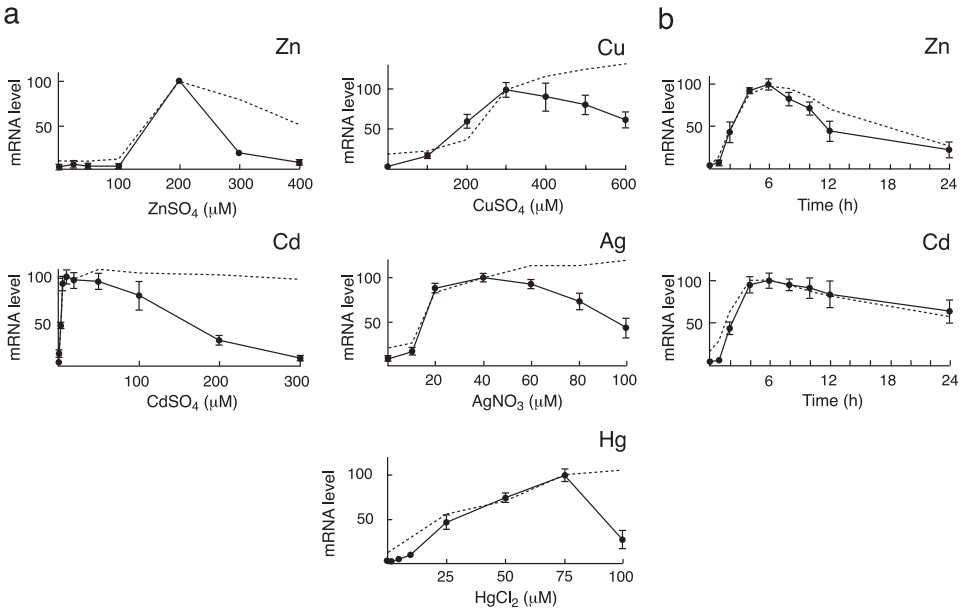

FIGURE 1. **Kinetics of *ho-1* expression induced by heavy metals.** *a*, dose-response curves. Levels of *ho-1* mRNA in HeLa cells exposed to indicated concentrations of $ZnSO_4$, $CdSO_4$, $CuSO_4$, $AgNO_3$, or $HgCl_2$ for 4 h were determined by northern blotting. For each panel, average values from three to six independent experiments each performed in duplicate are shown with standard errors. Values relative to the maximal responses (taken as 100) are presented. Dose-response curves of *hsp70* mRNA (10) are indicated by *broken lines* for comparison: values relative to the *hsp70* mRNA levels at metal concentrations with the maximal *ho-1* responses (taken as 100) are presented. *b*, time course. Time-dependent changes in *ho-1* mRNA levels after exposure to 200 μM $ZnSO_4$ or 5 μM $CdSO_4$ are shown as in *a*. Values relative to the *ho-1* or *hsp70* mRNA levels at 6 h (with the maximal *ho-1* responses; taken as 100) are presented.

MATERIALS AND METHODS

Cell Culture—HeLa S3 cells (CCL 2.2) were cultured in Eagle's minimum essential medium supplemented with 10% calf serum under 5% CO_2 atmosphere. In experiments, streptomycin (100 μg/ml) and penicillin (100 units/ml) were added to the medium.

Northern Blotting—A digoxigenin-labeled probe specific to *ho-1* was prepared as follows. An 847-bp SphI fragment containing most of human *ho-1* coding region was cut out of a plasmid pHHO1 (a generous gift from Prof. S. Shibahara), blunt-ended, and cloned between blunt-ended EcoRI and PstI sites of a plasmid pSPT18 (Roche Applied Science). Using the resultant plasmid as a template, a digoxigenin-labeled *ho-1*-specific RNA probe was synthesized *in vitro* as previously described (10). Northern blotting was carried out as described (10). Briefly, cells were inoculated in 60-mm Falcon plastic dish (10^6 cells/4 ml of medium) and cultured for 3 days. Cells in duplicates were incubated with or without heavy metals for 4 h before harvest. Total RNA was extracted from the cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method. RNAs were electrophoresed in formaldehyde-1% agarose gel, transferred to charged nylon membrane, and UV-cross-linked. The *ho-1* mRNA was detected with the digoxigenin-labeled specific RNA probe.

Electrophoretic Mobility Shift Assay and Supershift Assay—Whole cell extracts (WCEs) were prepared from HeLa cells as previously described (10). Electrophoretic mobility shift assay (EMSA) was performed with the WCE and ^{32}P-labeled double-stranded oligonucleotide probes as reported (10). The nucleotide sequences of synthetic oligonucleotide probes and competitors used are shown in Figs. 2*a* and 5, *a* and *b*. In supershift assay, 1 μl of antibody solution was added to EMSA reaction mixtures after binding reaction, followed by incubation at 25 °C for 20 min prior to electrophoresis. Antibodies used were a rabbit anti-human MTF-1 antibody (15), a rabbit anti-human HSF1 antibody (Affinity Bioreagents), and a rabbit anti-human Sp1 antibody (Santa Cruz Biotechnology). For all results, reproducibility was confirmed by repeated experiments. The anti-MTF-1 antibody had been raised against a 19-amino acid synthetic peptide and affinity-purified (15), and the anti-HSF1 antibody was polyclonal antiserum raised against a whole molecule of recombinant HSF1. Such different properties of the antibodies appeared to result in a striking difference in the size of supershifted complex.

Plasmids for Transfection—A reporter plasmid pHSP70prCAT that carries *hsp70* sequence (−270 to +156) linked to the chloramphenicol acetyltransferase (CAT) reporter gene and its variant pΔ(−112)CAT in which the sequence from −270 to −113 had been deleted were previously described (17). The latter retains HSE located between −105 and −91. Another variant pΔ(−76)CAT was constructed as follows. A plasmid pHSP70pr that carries *hsp70* sequence (from −270 to +156) was reported previously (17). In this construct, a KpnI site had been introduced just 5′ of the *hsp70* sequence, and a native BamHI site is present at its 3′ end. A DNA fragment was amplified by PCR with pHSP70pr as a template and the following primer pair: 5′-TGGGTACCAGATCTAGCTCGGTGATTGGCTCAGAAGG-3′ (forward) and 5′-TCCTCAGGCTAGCCGTTATCCG-3′ (reverse). The forward primer carries KpnI and BglII sites followed by *hsp70* promoter sequence between −76 and −54. The reverse primer carries the NheI site and flanking sequences that are located just downstream from TIS of *hsp70*. The resultant PCR product was digested with KpnI and NheI, yielding a fragment containing *hsp70* sequence form −76 to +7. A KpnI/NheI fragment from the original pHSP70pr containing *hsp70* sequence from −270 to +7 was replaced with the amplified truncated fragment, generating pΔ(−76) that lacks HSE but retains the promoter sequence up to −76. The KpnI/BamHI fragment from pΔ(−76) was cloned between the KpnI and BglII sites in the multicloning site of pCAT3 Basic (Promega) to place CAT reporter gene under the control of the truncated *hsp70* promoter, generating pΔ(−76)CAT.

Double-stranded oligonucleotides each containing the HSE or CdRE sequence with flanking 5′-BamHI and 3′-BglII sites were prepared; their sequences are as follows (only the upper strand sequences are shown; the flanking restriction enzyme sites

are indicated by lowercase letters): HSE, 5′-gatccAACCCCTGGAATATTCCCGACCTGGCAGCCa-3′; CdRE, 5′-gatccAGGCGGATTTTGCTAGATTTTGCTGAGTCAa-3′. These oligonucleotides were inserted into the BglII site of pΔ(−76)CAT (just upstream of *hsp70* promoter sequence) to generate p(HSE)CAT, p(CdRE)CAT and p(CdRE^r)CAT where the superscript "r" stands for reverse orientation. In p(HSE)CAT, HSE was placed exactly at the same position as native HSE relative to TIS. The double-stranded CdRE oligonucleotide was also inserted into the BamHI site located downstream of the CAT reporter gene to generate p(3′-CdRE)CAT and p(3′-CdRE^r)CAT. Mutant CdRE oligonucleotides with base substitutions corresponding to the mutants c, d, and g in Fig. 5*b* were also inserted into the BglII site of pΔ(−76)CAT, generating p(mut-c)CAT, p(mut-d)CAT, and p(mut-g)CAT, respectively. For all constructs, DNA sequences were confirmed by nucleotide sequencing.

A reference plasmid pRSV-βGal expresses β-galactosidase (βGal) under the control of Rous sarcoma virus long terminal repeat promoter (15). A HSF1 expression vector pCI-HSF1 expresses human HSF1 under the control of the human cytomegalovirus major immediate-early gene enhancer/promoter (17). For transient transfection assays, plasmid DNAs were purified using Qiagen plasmid maxi kit.

Transient Transfection Assay—HeLa S3 cells were plated at 2×10^5 cells/2 ml medium/35-mm Falcon plastic dish. After a 24-h incubation, cells were transfected with reporter, reference, and expression plasmids according to a standard calcium phosphate transfection protocol. Control cells without an expression vector were transfected with an equimolar amount of empty vector pCI (Promega). The amount of DNA to be transfected was adjusted to be 2 μg/dish in total by adding pUC19 if necessary. After appropriate treatments, CAT and βGal levels in cell extracts were determined using enzyme-linked immunosorbent assay kits (Roche Applied Science). CAT expression levels were normalized relative to βGal expression levels.

RESULTS

Human ho-1 and hsp70 Genes Show Similar Heavy Metal Responses—The responses of human *ho-1* gene expression to several heavy metals were examined in HeLa S3 cells. The results of Northern blot analysis are shown in Fig. 1*a*. *ho-1* was responsive to various metal salts including $ZnSO_4$, $CdSO_4$, $CuSO_4$, $AgNO_3$, and $HgCl_2$. We noticed that the metal response of *ho-1* was quite similar to that of *hsp70* observed in our earlier study (10). In the dose-response curves of *ho-1* expression in cells exposed to these metals, mRNA accumulation up to maximal induction was very similar in both genes (Fig. 1*a*; compare with *hsp70* curves represented by *broken lines*). At higher metal concentrations, *ho-1* mRNA levels decreased more sharply, in contrast to *hsp70* mRNA levels that were sustained. For zinc and cadmium, time course of *ho-1* induction was also examined at concentrations that induced maximal responses, *i.e.* at 200 and 5 μM, respectively (Fig. 1*b*). Time-dependent changes of *ho-1* mRNA accumulation in response to both metals were quite similar to those of *hsp70 mRNA*. Such a high extent of similarity in induction kinetics suggested that a common mechanism of transcriptional activation might be shared by these two genes.

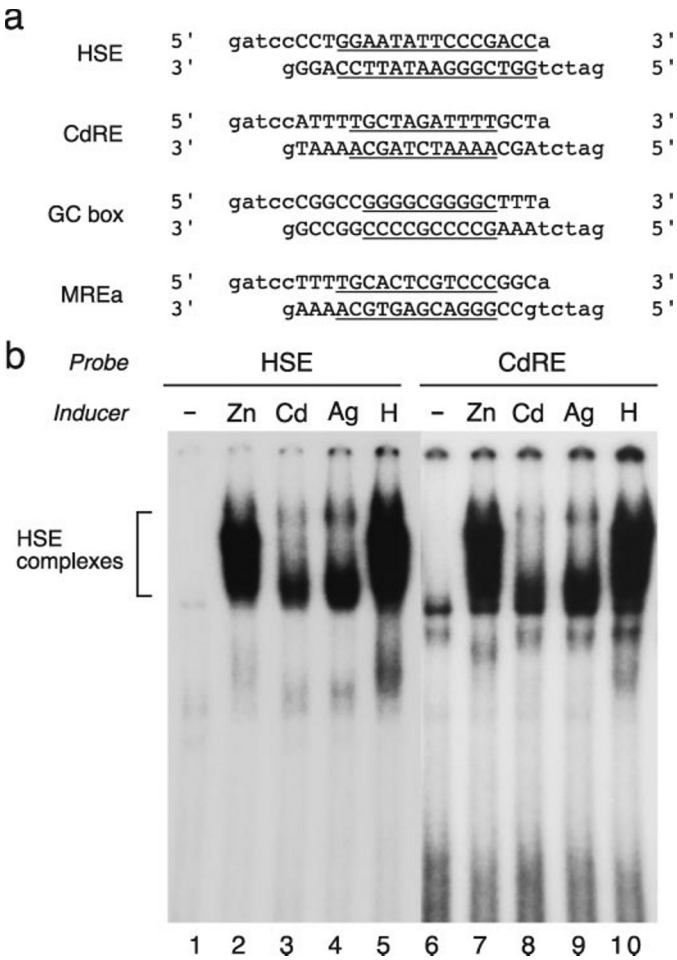

FIGURE 2. **HSE- and CdRE-binding proteins induced by heavy metals.** *a*, oligonucleotides used as probes and competitors in EMSA. Nucleotide sequences of double-stranded oligonucleotides used in EMSA experiments (Figs. 2–5) are shown. Nucleotides corresponding to each regulatory element are indicated by *underlines*: HSE from human *hsp70* (13), CdRE form human *ho-1* (2), and GC box (23) and MREa (7) from human *MT-2A*. Flanking restriction enzyme sites are indicated by *lowercase letters*. *b*, analysis of HSE- and CdRE-binding proteins of HeLa cells by EMSA. WCEs were prepared from HeLa cells incubated for 4 h with 200 μM $ZnSO_4$ (*lanes 2* and *7*), 10 μM $CdSO_4$ (*lanes 3* and *8*) or 50 μM $AgNO_3$ (*lanes 4* and *9*), or cells heat shocked at 43 °C for 2 h (*lanes 5* and *10*). WCEs were assayed by EMSA with a ^{32}P-labeled HSE (*lanes 1–5*) or CdRE (*lanes 6–10*) probe. Six μg of protein/lane was subjected to electrophoresis. *Lanes 1* and *6* represent untreated controls. The position of HSF1-HSE complexes is indicated by a *bracket*. Images in *lanes 1–5* are from x-ray film exposed for 17 h, while those in *lanes 6–10* from x-ray film exposed for 3 days.

HSF1 Specifically Binds to CdRE—It has been suggested that the metal response of *hsp70* is mediated by a transcription factor HSF1 which plays a primary role in transcriptional activation of *hsp* genes by heat shock. DNA binding activity of human HSF1 is activated by metal exposure, and the activated protein binds to HSE located ~100 bp upstream from TIS (10, 13). On the other hand, it has been reported that CdRE located ~4 kbp upstream from TIS of human *ho-1* mediates cadmium response of the gene (2). By EMSA with HSE and CdRE probes indicated in Fig. 2*a*, we analyzed HeLa cell extracts for protein factors that specifically recognize HSE or CdRE. As reported previously (10), indistinct HSE binding activities, which probably represent HSF1, were induced in WCE from cells exposed to zinc, cadmium, silver, or heat shock (Fig. 2*b*, *lanes 2–5*, respectively;

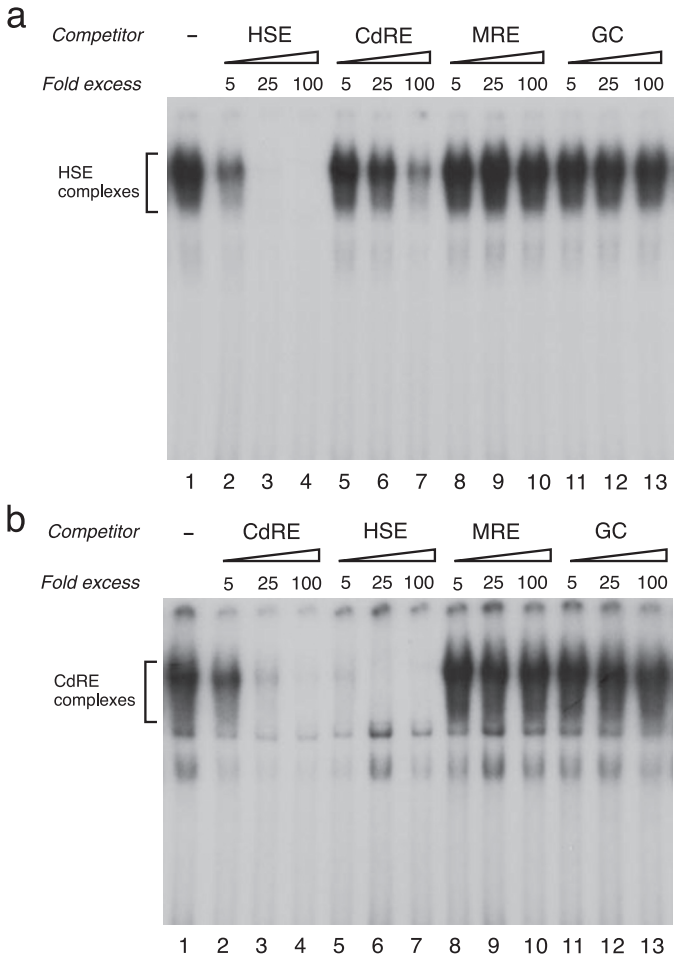

FIGURE 3. **Sequence recognition specificity of the HSE- and CdRE-binding proteins.** *a*, competitive EMSA with the ^{32}P-HSE probe. WCE was prepared from HeLa cells incubated with 200 μM $ZnSO_4$ for 4 h. EMSA binding reactions were performed in the presence of indicated amounts (5–100-fold excess over the probe) of unlabeled competitor oligonucleotides: HSE (*lanes 2–4*), CdRE (*lanes 5–7*), MRE (*lanes 8–10*), or GC-box (*lanes 11–13*). *Lane 1* represents control without competitors. Four μg of protein/lane was subjected to electrophoresis. HSE complexes are indicated by a *bracket*. *b*, competitive EMSA with the ^{32}P-CdRE probe. Assays were performed as in a with ^{32}P-CdRE probe and indicated amounts of unlabeled competitor oligonucleotides: CdRE (*lanes 2–4*), HSE (*lanes 5–7*), MRE (*lanes 8–10*), or GC box (*lanes 11–13*). *Lane 1* represents control without competitors. Eight μg of protein/lane was subjected to electrophoresis. CdRE complexes are indicated by a *bracket*.

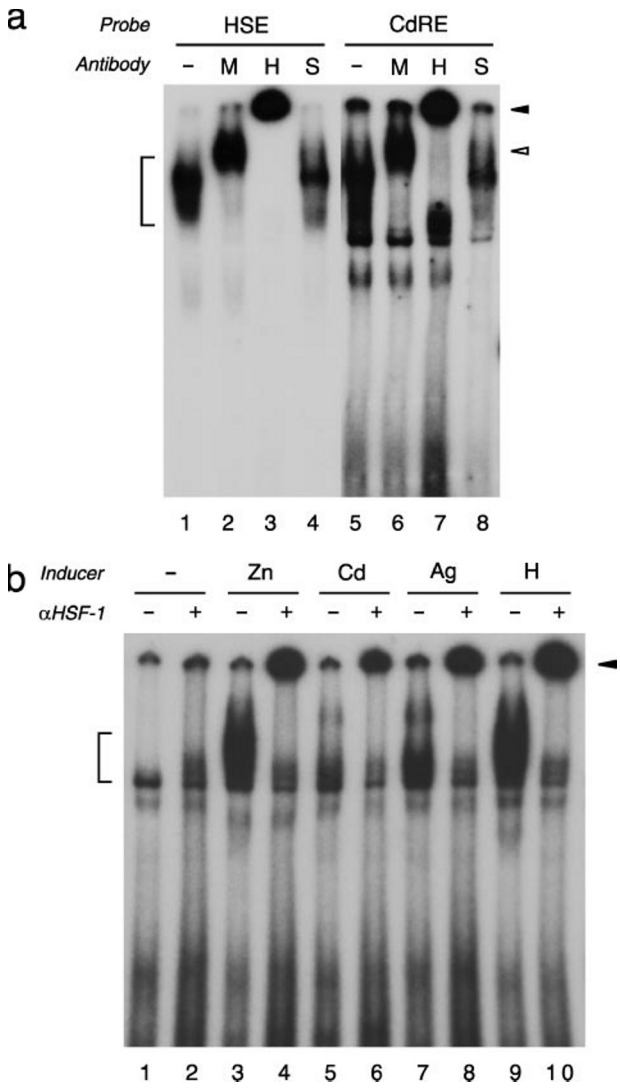

FIGURE 4. **Identification of CdRE-binding proteins.** *a*, supershift analysis of zinc-induced HSE and CdRE complexes. WCE was prepared from HeLa cells incubated with 200 μM $ZnSO_4$ for 4 h. EMSA binding reactions were performed with the WCE and the ^{32}P-HSE (*lanes 1–4*) or ^{32}P-CdRE (*lanes 5–8*) probe. The reaction mixtures were then incubated without (*lanes 1* and *5*) or with an antibody raised against MTF-1 (*M*; *lanes 2* and *6*), HSF1 (H; *lanes 3* and *7*) or Sp1 (S; *lanes 4* and *8*) before electrophoresis (7.5 μg of protein/lane). HSF1-DNA complexes are indicated by a *bracket*. The *black arrowhead* indicates the position of the supershifted band by the anti-HSF1 antibody. The *white arrowhead* indicates the supershifted band by the anti-MTF-1 antibody. Images in *lanes 1–4* are from x-ray film exposed for 16 h, while those in *lanes 5–8* from x-ray film exposed for 3 days. *b*, supershift analysis of CdRE complexes induced by various treatments. WCEs were prepared from HeLa cells incubated for 4 h in the absence (*lanes 1* and *2*) or presence of 200 μM $ZnSO_4$ (*lanes 3* and *4*), 10 μM $CdSO_4$ (*lanes 5* and *6*), or 50 μM $AgNO_3$ (*lanes 7* and *8*) or cells heat shocked at 43 °C for 2 h (*lanes 9* and *10*). After EMSA binding reaction with the WCEs and the ^{32}P-CdRE probe, the reaction mixtures were incubated without (*lanes 1*, *3*, *5*, *7*, and *9*) or with the anti-HSF1 antibody (*lanes 2*, *4*, *6*, *8*, and *10*) before electrophoresis (6 μg of protein/lane). CdRE complexes are indicated by a *bracke*t. The *arrowhead* indicates the position of supershifted bands.

compare with control in *lane 1*). CdRE binding activities were also observed to be induced by these heavy metals as well as heat shock (*lanes 7–10*). Surprisingly, these CdRE-protein complexes were indistinguishable in mobility with the HSE-protein complexes, although signals were less intense. From these results, it seemed likely that the CdRE-binding protein is HSF1.

Specificity of sequence recognition by the HSE or CdRE binding activity was analyzed by competitive EMSA (Fig. 3). In an assay with WCE from zinc-exposed cells and ^{32}P-HSE probe (Fig. 3*a*), the ^{32}P complexes were competed out by unlabeled HSE oligonucleotide in a dose-dependent manner (*lanes 2–4*; compare with control without competitor in *lane 1*). Unlabeled CdRE oligonucleotide was observed to be also competitive, although less efficient than the HSE competitor (*lanes 5–7*). By contrast, no competition was observed with oligonucleotide competitors containing MRE (*lanes 8–10*) or GC box (*lanes 11–13*), the recognition sequences of transcription factors MTF-1 and Sp1, respectively. In another assay with ^{32}P-CdRE probe (Fig. 3*b*), the ^{32}P complexes were competed out by the CdRE oligonucleotide (*lanes 2–4*) and also by the HSE oligonucleotide more efficiently (*lanes 5–7*). MRE (*lanes 8–10*) and GC oligonucleotides (*lanes 11–13*) again showed no effect. From these results, an identical protein, probably HSF1, appears to recognize both HSE and CdRE but with higher affinity to HSE.

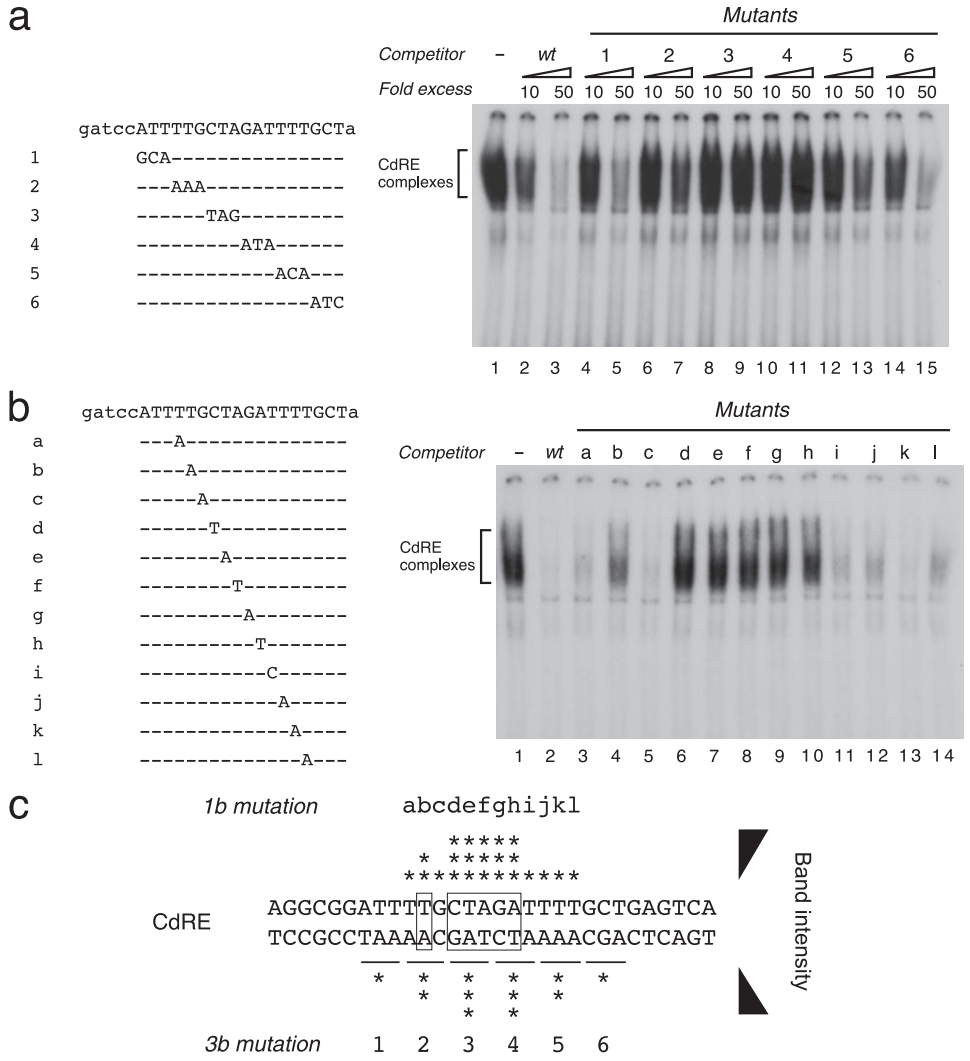

FIGURE 5. **Identification of bases essential for HSF1-CdRE interaction.** *a*, competitive EMSA with a series of mutant CdRE competitors with consecutive 3-base mutations. The upper strand sequence of wild type CdRE (*wt*) and substituted bases in mutants 1–6 are indicated on the *left*. Flanking restriction enzyme sites are indicated by *lowercase letters*. EMSA reaction was performed with WCE from HeLa cells incubated with 200 μM $ZnSO_4$ for 4 h and the ^{32}P-CdRE probe in the absence or presence of indicated amounts (10- or 50-fold excess) of competitors, followed by electrophoresis (8 μg of protein/lane). CdRE complexes are indicated by a *bracket*. *b*, competitive EMSA with a series of mutant CdRE competitors with single base mutations. Wild type CdRE sequence (*wt*) and substituted bases in mutants a-l are indicated as described for *a*. EMSA reaction was performed as described for *a*, in the absence or presence of a 50-fold excess of competitors, followed by electrophoresis (6 μg of protein/lane). *c*, summary of the results. Band intensity of the CdRE complexes from each reaction in *a* and *b* are indicated by the number of *asterisks* below and above the CdRE sequence, respectively. Higher band intensity means lower affinity to HSF1. Bases important for HSF1 binding are indicated by *rectangles*.

To identify the CdRE-binding protein, zinc-induced protein-DNA complexes were further analyzed by supershift assays with specific antibodies (Fig. 4*a*). When an EMSA binding reaction mixture with ^{32}P-HSE probe was incubated with an anti-HSF1 antibody prior to electrophoresis, the HSE complexes were supershifted, indicating the formation of a huge complex electrophoresed only a small distance into gel (*lane 3*; compare with control without antibody in *lane 1*). The CdRE complexes were also supershifted by this antibody exactly in the same manner (*lane 7*; compare with control without antibody in *lane 5*). These results strongly suggested that HSF-1 was contained in the CdRE complexes. We have previously reported possible involvement of MTF-1 in the HSF1-HSE complexes (15). An anti-MTF-1 antibody raised against a synthetic peptide containing a part of MTF1 sequence supershifted the CdRE complexes (*lane 6*) as well as the HSE complexes (*lane 2*), suggesting that MTF-1 is also contained in the CdRE complexes. An anti-Sp1 antibody used as a negative control supershifted neither HSE complexes (*lane 4*) nor CdRE complexes (*lane 8*), verifying specific supershift by anti-HSF1 and anti-MTF-1 antibodies. CdRE complexes induced by cadmium, silver, and heat shock (Fig. 4*b*, *lanes 6*, *8*, and *10*; compare with controls without a specific antibody in *lanes 5*, *7*, and *9*, respectively) were supershifted in the same manner as zinc (*lane 4*) by the anti-HSF1 antibody, indicating the involvement of HSF in any of these complexes. From these data, it is likely that heavy metal exposure activates HSF1 to be able to bind CdRE, through which HSF1 plays a role in the heavy metal regulation of *ho-1*.

Nucleotides Required for the Recognition of CdRE by HSF1—Bases essential for the recognition of CdRE by HSF1 were examined by competitive EMSA using various mutant CdRE oligonucleotides as unlabeled competitors (Fig. 5). When a series of mutants with substitutions in three consecutive bases was used as competitors (Fig. 5*a*), mutant 3 (*lanes 8* and *9*) and mutant 4 (*lanes 10* and *11*) that had substitutions at the center of the CdRE sequence CTAGAT lost the ability to competitively inhibit CdRE complex formation (compare with controls of the wild type CdRE competitor in *lanes 2* and *3*), indicating that these bases are particularly important. Decreased competition was observed for mutant 2 (*lanes 6* and *7*) and mutant 5 (*lanes 12* and *13*), which had substitutions next to the central bases. Mutant 1 (*lanes 4* and *5*) and mutant 6 (*lanes 14* and *15*) showed no effect. We then tested another series of CdRE mutant competitors, each of which had a single base change within the sequence TTGCTAGATTTT found to be important in Fig. 5*a*. As shown in Fig. 5*b*, the ability to compete with CdRE was lost in mutants d, e, f, g, and h (*lanes 6–10*) and was decreased in mutant b (*lane 4*) relative to wild type CdRE (*lane 2*). Other base changes did not affect HSF1 binding. The results of Fig. 5, *a* and *b*, are summarized in Fig. 5*c*. In conclusion, the base sequence essential for HSF1 binding is TnCTAGA.

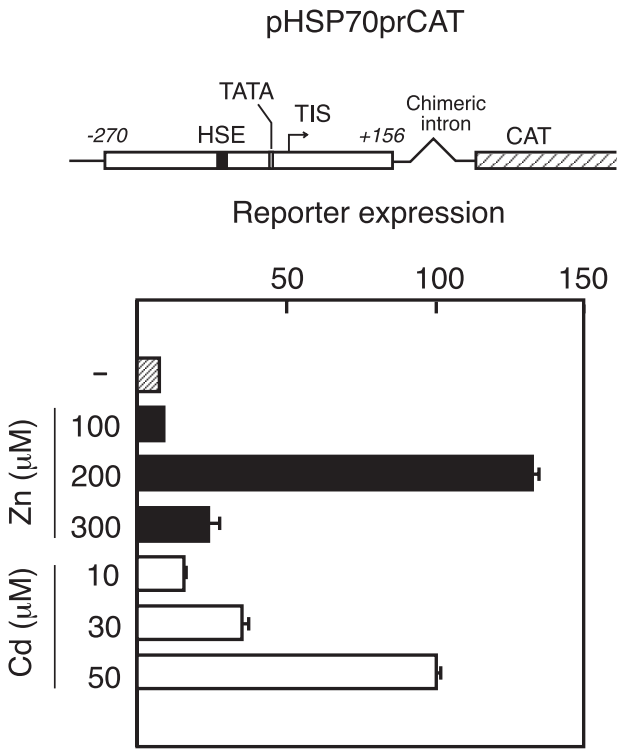

FIGURE 6. **Transient transfection assay that detects heavy metal responses of *hsp70* promoter.** HeLa cells were inoculated at 2×10^5 cells/2 ml medium/35-mm dish and incubated for 24 h. The cells were transfected with a reporter plasmid pHSP70prCAT (0.6 μg; structure is schematically shown at the *top*) and a reference plasmid pRSV-βGal (0.03 μg). After 24 h, the cells were incubated without or with $ZnSO_4$ (100, 200, or 300 μM) or $CdSO_4$ (10, 30, or 50 μM) for further 6 h. CAT and βGal levels in cell extracts were determined, and CAT values were normalized relative to βGal values. Averages from three independent experiments each performed in duplicate are shown with standard errors. Values relative to that obtained for 50 μM cadmium-treated cells (taken as 100) are presented. *Hatched bar*, uninduced control cells; *black bars*, zinc-induced cells; *white bars*, cadmium-induced cells.

CdRE Mediates HSF1-dependent Transcription—We next examined whether CdRE mediates transcriptional activation in a HSF1-dependent manner by transient transfection analysis. First, we attempted to establish an assay system that can detect heavy metal-induced transcription, using the regulatory region of *hsp70* that has a functional HSE and is known to be responsive to cadmium through HSF1-HSE interaction (3, 10, 13). We avoided using the widely used luciferase gene in this assay, since we have noted that luciferase enzyme activity used as a reporter is often affected by heavy metals, leading to misinterpretation of data. Alternatively, we have verified that the determination of CAT or βGal levels by enzyme-linked immunosorbent assay, which was adopted in the present work, can circumvent this difficulty.[4] A plasmid construct that carries *hsp70* upstream sequence linked to CAT reporter gene (pHSP70prCAT; indicated at the top of Fig. 6) was used as a reporter plasmid. As a reference plasmid, pRSV-βGal that expresses βGal was used. Levels of the reporter gene expression were determined after exposure to 100–300 μM $ZnSO_4$ or 10–50 μM $CdSO_4$ (Fig. 6). Increased expression of the reporter gene in a manner dependent on heavy metal concentrations was observed for both metals, demonstrating that this assay can successfully detect metal response.

We then tested variants of pHSP70prCAT that retained or lacked HSE located between −105 and −91 to examine whether HSE-dependent transcription is detectable. In a deletion mutant pΔ(−112)CAT, the 5′-flanking region of *hsp70* from −113 to −270 was deleted, but HSE was retained. In another mutant pΔ(−76)CAT, the 5′-flanking region of *hsp70* from −77 to −270 including HSE was deleted. We estimated reporter expression from these constructs by the transfection assay described above, but only low levels of activity were detected, whether HSE was retained or not (data not shown). This may indicate that the retained HSE is insufficient for mediating metal-responsive transcription. However, we found that HSE-dependent transcription was detectable under a different assay condition, that is, in the presence of overexpressed recombinant HSF1. Co-transfection of a HSF1 expression vector markedly enhanced basal level activity of pHSP70prCAT and almost abolished metal response (Fig. 7). Such an effect of HSF1 overexpression has also been observed in induction by zinc (17), probably due to titrating out negative regulators of HSF1 such as HSP90 and HSP70 (18, 19). Under this condition, pΔ(−112)CAT retaining HSE showed obvious reporter expression, in contrast to pΔ(−76)CAT lacking HSE which had almost no activity (Fig. 7). These results indicated that this assay was useful to detect HSF1-dependent transcriptional activity. Next we constructed two reporter plasmids based on pΔ(−76)CAT, p(HSE)CAT and p(CdRE)CAT, in which a synthetic oligonucleotide containing HSE or CdRE sequence was inserted just upstream of the regulatory sequence of pΔ(−76)CAT, respectively (Fig. 7). The former had HSE placed at exactly the same distance from TIS as native HSE and served as a positive control for p(CdRE)CAT in which the HSE sequence was replaced with CdRE sequence. Activity similar to that of pΔ(−112)CAT was observed for p(HSE)CAT. Expression from p(CdRE)CAT was also evident, even a little higher than that from p(HSE)CAT. These findings demonstrated that CdRE can mediate HSF1-dependent transcription. In the absence of overexpressed HSF1, only a background level of activity was observed for p(HSE)CAT or p(CdRE)CAT (data not shown).

To examine whether the HSF1-dependent transcriptional activity of CdRE requires direct binding of HSF1 to it, reporter plasmids carrying mutant CdREs were assayed (Fig. 8). The CdRE sequence in p(CdRE)CAT was replaced with mutant CdREs each with a base substitution corresponding to mutant c, d, or g shown in Fig. 5*b*: mutant c can bind HSF1 just like wild type CdRE, while mutants d and g cannot. Reporter plasmids with CdRE competent for HSF1 binding, *i.e.* wild type CdRE and mutant c, showed high reporter expression. By contrast, reporter activity was markedly decreased to near the background level in mutants d and g that do not bind HSF1, suggesting that direct binding of HSF1 to CdRE is essential for transcriptional activation.

To examine whether the activity of CdRE depends on orientation or location, several additional reporter plasmids were constructed and tested for expression (Fig. 9). The CdRE sequence was inserted in a reverse orientation relative to that in

[4] S. Koizumi and K. Suzuki, unpublished observation.

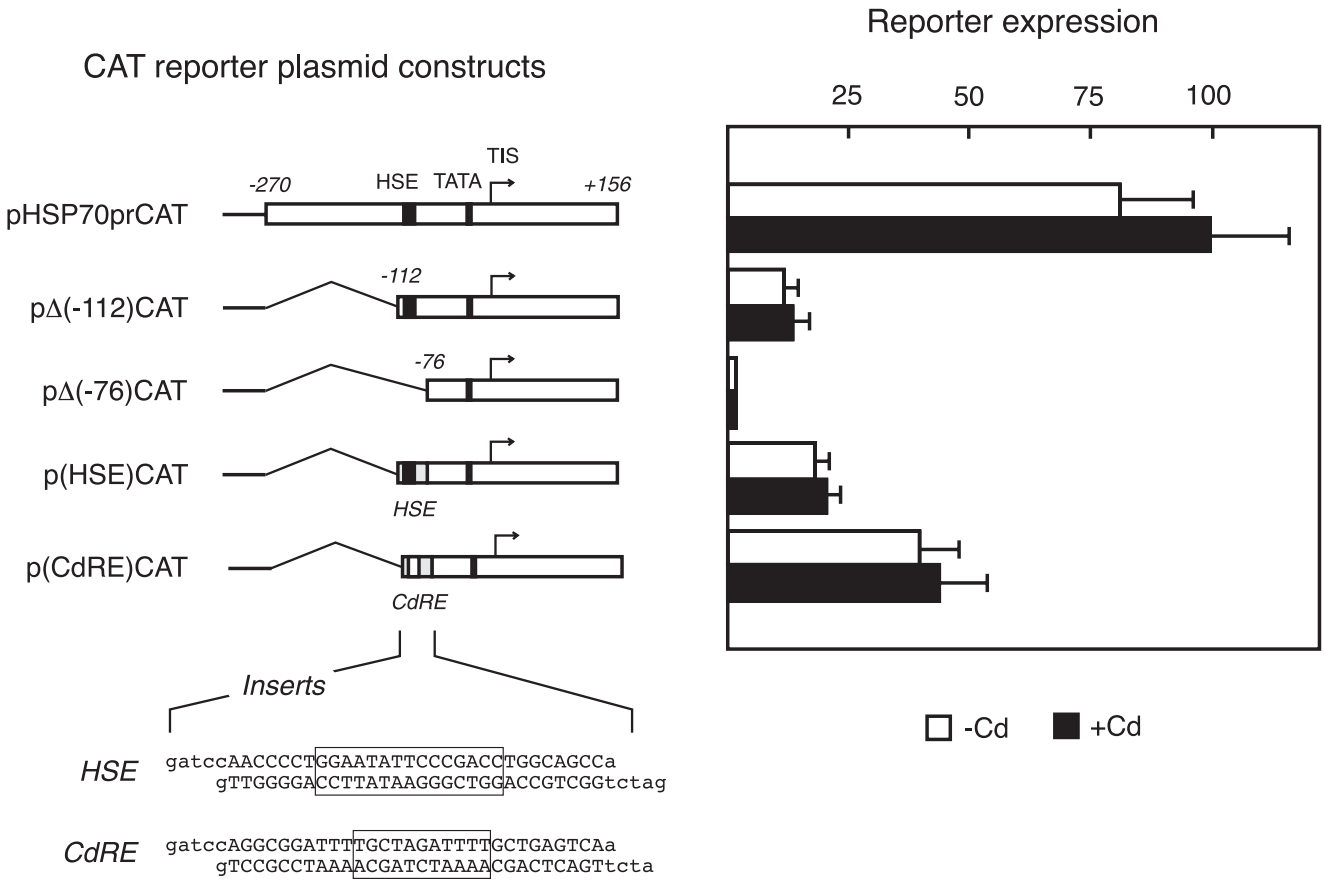

FIGURE 7. **CdRE mediates HSF1-dependent transcriptional activation.** The regulatory sequences of CAT reporter plasmid constructs used are schematically shown on the *left*. The wild type pHSP70prCAT carried 5′-flanking sequence of *hsp70* up to −270. Deletion mutants Δ(−112)CAT and Δ(−76)CAT lacked sequences −270 to −113 and −270 to −77, respectively; the former retained, but the latter lacked, HSE. p(HSE)CAT and p(CdRE)CAT had oligonucleotide inserts containing *hsp70* HSE or *ho-1* CdRE (sequences indicated at the *bottom*) at the 5′ end of the regulatory sequence of pΔ(−76)CAT, respectively. HeLa cells were transfected with a reporter plasmid (0.6 μg of pHSP70prCAT or equimolar amounts of others), pRSV-βGal (0.03 μg) and a HSF1 expression vector pCI-HSF1 (0.1 μg). After 24 h, $CdSO_4$ was added to medium to 30 μM (*black bars*) or not (*white bars*), and incubation was continued for further 6 h. Levels of reporter expression were determined as in Fig. 6. Averages from three independent experiments each performed in duplicate are shown with standard errors. Values relative to that obtained for cadmium-induced pHSP70prCAT (taken as 100) are presented.

p(CdRE)CAT, generating p(CdRE^r) CAT. This plasmid showed an activity comparative with p(CdRE)CAT. Alternatively, CdRE was inserted just 3′ of the reporter gene in the normal or reverse orientation, yielding p(3′-CdRE) CAT and p(3′-CdRE^r) CAT, respectively. These plasmid showed low but significant reporter expression (2.5–3-fold over the background level) as compared with control pΔ(−76)CAT, regardless of orientation. These results confirmed that CdRE possesses a HSF1-dependent enhancer-like activity.

DISCUSSION

CdRE is a regulatory DNA element located in the upstream region of human *ho-1* and has been reported to mediate cadmium-induced transcriptional activation (2). Although metal response via CdRE appears to be an excellent model for studying biological responses to metals, its mechanistic background has not been well characterized. In the present study, we showed that (*a*) HSF1, which plays an essential role in heat shock response of *hsp* genes, is activated by several heavy metals to be competent for DNA binding, (*b*) activated HSF1 specifically binds to CdRE, (*c*) CdRE mediates activation of transcription from a downstream promoter in the presence of overexpressed HSF1, and (*d*) this activation requires direct binding of HSF1 to CdRE and can also be mediated by CdRE placed in a reverse orientation or from a distance. The similarity in the heavy metal response observed between *hsp70* and *ho-1* might reflect the interaction of upstream regulatory elements with a common regulator HSF1. The finding that HSF1 is directly involved in the regulation of *ho-1*, which plays an important role in protection against oxidative stress (20), revealed an unexpected cross talk in the regulation of heavy metal-inducible genes, a new aspect of the biological defense system.

The nucleotide sequence of CdRE, TGCTAGATTTT, as well as the bases recognized by HSF1, TnCTAGA, appears to be unrelated with the typical HSE sequence. However, the sequence of CdRE and flanking regions, GGATT-TTGCT-AGATT-TTGCT, is composed of four inverted pentanucleotide repeats similar to the conserved nGAAn units in HSE (Ref. 3; units are indicated by separating with *hyphens*; bases recognized by HSF1 are underlined). In each unit, the second base (G of nGAAn) is conserved. Mutations that abolished HSF1 binding are located in the central two units, which are expected to be indispensable for binding of an active HSF1 trimer. Based on this consideration, CdRE may be regarded as a variant of HSE. Nevertheless, the central TA in the TnCTAGA sequence are most important for HSF1 binding from our results, despite of

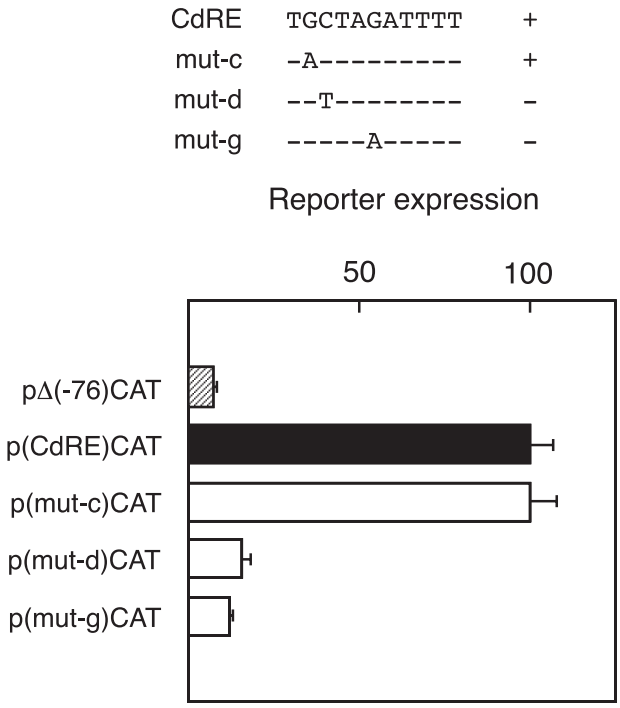

FIGURE 8. **Direct binding of HSF1 is essential for CdRE function.** The CdRE sequence in p(CdRE)CAT was replaced with mutant CdRE sequences each carrying a base substitution corresponding to mutant c, d, or g in Fig. 5*b*, yielding p(mut-c)CAT, p(mut-d)CAT, and p(mut-g)CAT, respectively. The substituted bases and the ability of each mutant to bind HSF1 are shown at the *top*, based on the results of Fig. 5*b*. HeLa cells were transfected with a reporter plasmid (0.6 μg of p(CdRE)CAT or equimolar amounts of others), pRSV-βGal (0.03 μg) and pCI-HSF1 (0.1 μg), followed by an incubation for 30 h. Levels of reporter expression were determined as described for Fig. 6. Averages from three independent experiments each performed in duplicate are shown with standard errors. Values relative to that obtained for p(CdRE)CAT (taken as 100) are presented. *Hatched bar*, control reporter plasmid without CdRE; *black bar*, wild type p(CdRE)CAT; *white bars*, reporter plasmids with mutant CdRE sequences.

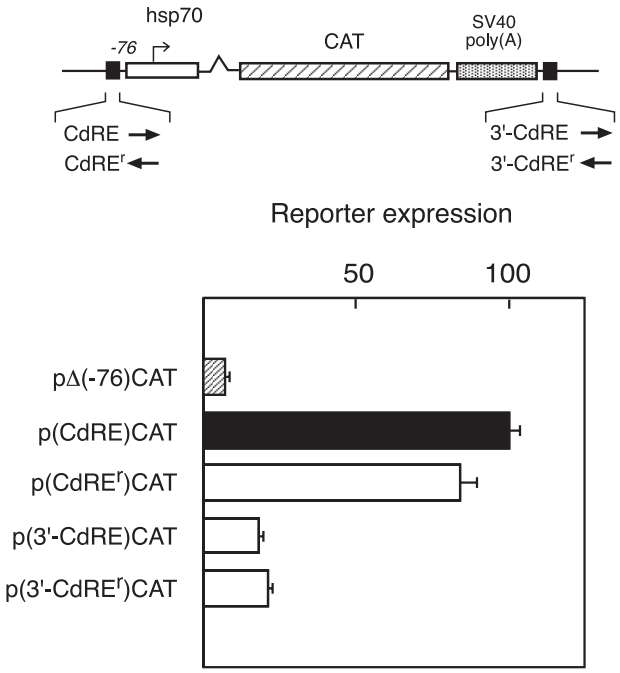

FIGURE 9. **CdRE possesses HSF1-dependent enhancer-like activity.** Variants of p(CdRE)CAT were assayed for HSF1-dependent transcriptional activity: p(CdRE^r)CAT with CdRE in a reverse orientation and p(3′-CdRE)CAT and p(3′-CdRE^r)CAT with CdRE at the 3′ end of the CAT reporter gene in a normal or reverse orientation, respectively. The positions of CdRE are schematically shown at the *top*. HeLa cells were transfected with a reporter plasmid (0.6 μg of p(CdRE)CAT or equimolar amounts of others), pRSV-βGal (0.03 μg), and pCI-HSF1 (0.1 μg), followed by an incubation for 30 h. Levels of reporter expression were determined as described for Fig. 6. Averages from four independent experiments each performed in duplicate are shown with standard errors. Values relative to that obtained for p(CdRE)CAT (taken as 100) are indicated. *Hatched bar*, control reporter plasmid without CdRE; *black bar*, wild type p(CdRE)CAT; *white bars*, reporter plasmids with differentially arranged CdRE.

the fact that the two bases correspond to non-conserved bases in the HSE consensus. Therefore it remains unclear whether HSF1 interacts with CdRE exactly in the same manner as with HSE. CdRE showed lower affinity to HSF1 in EMSA (Fig. 3), while exhibited higher activity in the transfection assay (Fig. 7), than HSE. These findings may possibly reflect a differential contact of HSF1 and CdRE, which would affect the conformation of the HSF1 molecule and subsequent interaction with other regulatory proteins such as coactivators.

As described above, CdRE binding activity of HSF1 is induced in the extracts of heavy metal-treated cells, and CdRE displays a HSF1-dependent enhancer-like activity. These findings make us imagine that binding of metal-activated HSF1 to CdRE is involved in the mechanism of transcriptional activation of *ho-1*. However, CdRE by itself was not sufficient for transcriptional activation *in vivo*. In our transient transfection assay, CdRE exhibited an obvious transcriptional activity only in the presence of overexpressed HSF1, whereas not in its absence, namely in transcription utilizing only the endogenous HSF1. Without exogenous HSF1, our transfection assay can detect heavy metal-induced transcription from *hsp70* promoter, for which the interaction of HSF1 and an upstream HSE is critical (3, 10, 13). Therefore at least in transcription directed by the 5′-flanking sequence of *hsp70*, activation, nuclear translocation, and binding to HSE of the endogenous HSF1 must occur. The fact that the isolated CdRE sequence was not functional under the same condition indicates that CdRE by itself is insufficient for mediating metal-induced transcription, and additional regulatory sequences might be required for CdRE to be fully functional. Although HSF1 showed an obvious CdRE binding activity in EMSA, the protein-DNA interaction may not be stable enough *in vivo*, and the weak interaction might be compensated only by a high concentration of HSF1.

Based on the findings described above, it seems important to identify sequences that cooperate with CdRE to achieve full activity. It has been reported that STAT-1 (signal transducer and activator of transcription-1) that bind to a region near the HSF1 binding site cooperates with HSF1 via direct protein-protein interaction to produce strong transcriptional activation (21). Such a regulator binding site near CdRE is a possible candidate for the additional sequence to assist CdRE function. In this context, it is of interest that a Maf recognition element (MARE) is located just adjacent to CdRE (22). It has been suggested that nuclear export of a negative regulator Bach1 that binds to MARE is involved in cadmium-induced transcriptional activation of human *ho-1* (12), although it is not clear whether the MARE next to CdRE is directly concerned in such a regulation. In addition, it has recently been reported that a nucleolar protein pescadillo regulates transcription of *ho-1*

through binding to CdRE in renal cells (11). This protein is an additional new member to be studied in the regulation of *ho-1* via the CdRE/MARE region. Possible cooperation between CdRE and additional sequences including MARE, as well as the interaction of proteins binding to them, is now under investigation.

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