Heme oxygenase-1 (EC 1.14.99.3) is an essential enzyme in the physiologic heme catabolism and catalyzes the oxidative cleavage of heme at the α-methene bridge, resulting in the formation of carbon monoxide, iron, and biliverdin, an immediate precursor of bilirubin. The activity of heme oxygenase is high in the spleen, liver, and bone marrow, in which senescent erythrocytes are sequestered and destroyed by resident macrophages. Thus, heme oxygenase plays a key role in hemapoiesis in recruiting iron to erythroblasts in the bone marrow. Furthermore, expression of heme oxygenase-1 mRNA is noticeably increased during differentiation of myelomonocytic cell lines treated with various reagents, including 12-O-tetradecanoylphorbol-13-acetate (TPA) and the active form of vitamin D₃. In this context, we showed that transcription of the heme oxygenase-1 gene is activated during differentiation of a human monocytic leukemia cell line THP-1 to macrophages. Increased activity of heme oxygenase-1 may represent the functions of activated macrophages, such as breakdown of heme derived from phagocytosed erythrocytes. Alternatively, carbon monoxide and ferric iron produced may be involved in bacteriocidal action through the generation of active oxygen intermediates.

It is well known that heme oxygenase-1 activity is highly induced in human cell lines by its substrate heme and by various environmental derangements, such as heavy metals, UV light, and TPA. Thus, heme oxygenase-1 was established as a stress protein and, in particular, rat heme oxygenase-1 is a heat shock protein (HSP). The human heme oxygenase-1 gene promoter contains a sequence (positions -384 to -365) identical to a consensus sequence of heat shock element (HSE), termed HHO-HSE. HSE is the cis-regulatory element responsible for transcriptional activation of the HSP genes by high temperature and other stresses and was identified as a highly conserved palindromic sequence, 5'-CNGAANNTTCNNG-3', or as contiguous arrays of variable numbers of the 5-bp unit (5'-NGAAN-3') in alternating orientation. However, using transient expression assays, we previously reported that the human heme oxygenase-1 promoter containing HHO-HSE is unable to confer the heat-mediated induction of a reporter gene. Furthermore, the activity of heme oxygenase-1 was not induced by heat shock in several human cell lines, although heat shock led to a small increase in heme oxygenase-1 mRNA levels in HeLa cells and in skin fibroblasts. Especially in a human hepatoma cell line (Hep3B), heme oxygenase-1 mRNA levels were remarkably increased by heat shock.

In this study, we provide several lines of evidence that HHO-HSE is potentially functional, but that its function is repressed in certain cells.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from Takara Shuzo (Otsu, Japan), Boehringer Mannheim (Mannheim, Germany), and New England BioLab (Beverly, MA). Klönzym enzyme was purchased from Takara Shuzo. [α-32P]-dCTP and anti-HSP70 monoclonal antibodies were purchased from Amersham (Buckinghamshire, UK). [γ-32P]-ATP was from ICN (Costa Mesa, CA).

Isolation and treatment of human alveolar macrophages. The bronchoalveolar lavage fluid was filtered through sterile gauze and centrifuged at 600g for 5 minutes. The bronchoalveolar lavage cells were resuspended in Dulbecco’s modified Eagle medium and checked under a light microscope. This procedure yielded 1.2 × 10⁶ viable cells that consisted of 94% alveolar macrophages, 5% lymphocytes, and 1% polymorphonuclear leukocytes. The cells were washed again and resuspended in the same medium at a concentration of 10⁶ cells/mL. Cells (2.5 × 10⁶) were plated in a 50-mm diameter dish and incubated for 5 hours at 37°C or 42°C under 5%
CO₂. As a 0-time control, cells were harvested without incubation, immediately frozen in liquid nitrogen, and stored at -80°C.

**Cell culture.** YN-1-0-A, a human erythroblastoid cell line, is a clonal variant of YN-1 derived from a patient with blastic crisis of chronic myelogenous leukemia. Hemoglobin synthesis can be induced in YN-1-0-A cells by the treatment with hemin or cytokines arabinoside.  

YN-1-0-A cells were maintained in serum-free Iscove’s modified Dulbecco’s medium (IMDM) containing FeCl₃ (2.6 × 10⁻³ mol/L) as an additional requirement. Cells were cultivated at 37°C under 5% CO₂, unless otherwise indicated.

**Western blot analysis.** Western blot analysis was performed as described previously using antihuman heme oxygenase-1 polyclonal antibody or antihuman HSP70 monoclonal antibody. After incubation of alveolar macrophages for 5 hours, cell pellets were lysed for 1 hour at 4°C in the lysis buffer and centrifuged at 12,000g for 10 minutes. The supernatant obtained was mixed with loading buffer that was electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel (12%). In cases of YN-1-0-A, 4 × 10⁶ cells were incubated for 5 hours at 42°C or at 37°C in a 250-mL flask containing serum-free IMDM with or without 5 μmol/L hemin and harvested for Western blot analysis.

**Preparation of whole-cell extracts and gel mobility shift assays.** Whole-cell extracts were prepared by the method of Mosser et al from untreated YN-1-0-A cells or cells treated for 30 minutes at 42°C or at 37°C in the presence of 5 μmol/L hemin or 5 μmol/L cadmium (Cd). Whole-cell extracts were incubated with a DNA probe and the formation of protein-DNA complexes was analyzed as detailed previously. The end-labeled probes were used as the Dde I/Ban II fragment (−405/−315) and the Dde I/Sal I fragment (−405/−344), carrying HHO-HSE, and the Ava II/Taq I fragment (−147/−78) containing an HSE of the human HSP70 gene (HSP70 HSE). The numbers in parentheses, shown together with restriction enzymes, indicate the 5'-terminal nucleotide generated by cleavage.

**Construction of chimeric fusion genes.** The Ava II/HindIII fragment, containing the DNA segment (nucleotide residues −991 to 24) of the human heme oxygenase-1 gene and a part of the polylinker sequence of pUC8, was isolated from a subclone, SpHHO8. The single-stranded ends of this fragment were filled in and ligated to a HindIII linker. After digesting with HindIII and Sal I, the resulting HindIII (Ava II)/Sal I (Xho I) fragment (−991/20) was isolated. Using this procedure, the Ava II site (position −991) was converted to the HindIII site and is shown within parenthesis. The Xho I site at position 20 had been converted to the Sal I site by adding a linker sequence. This HindIII (Ava II)/Sal I (Xho I) fragment was then ligated to the larger fragment of pHHOgp2 linearized with HindIII and Sal I to replace the rat heme oxygenase-1 gene promoter with the human heme oxygenase-1 gene promoter. The Sal I site of pHHOgp2 is located at the junction between the rat heme oxygenase-1 gene and the Escherichia coli gpt coding for xanthine-guanine phosphoribosyltransferase. The parent plasmid pHHOgp3 thus obtained contains the 5'-flanking region and the 5'-portion of exon I of the human heme oxygenase-1 gene (−991 to 24) upstream of the gpt gene. A fusion gene pHHOgpO3/A4, lacking the poly(dG-dT) region, was constructed by deleting the Pat I/Msc I fragment (−282/−176) from pHHOgp3. A fusion gene pHHOgp4 contains the HindIII (Msc I)/Xho I fragment (−176/20) derived from pHHOgp3 upstream from the gpt gene. The synthetic HHO-HSE was inserted at the HindIII site of pHHOgp4, the site of which had been filled in by Klenow fragment, yielding pHHOgp4O. The fragments, f41/45, were prepared by digesting the 5'-flanking region of pHHOgp3 with the restriction enzymes indicated (see Fig 5). Each fragment was filled in by Klenow fragment, followed by blunt-end ligation to the HindIII site of pHHOgp4 (f41/45), the site of which had been filled in by Klenow fragment. The fusion piasmids, pHHOgp41/45, were thus obtained.

Another series of the fusion genes, pHHOL14, pHHOL15, and pHHOL5, contain the 5'-flanking region of the human heme oxygenase-1 gene upstream from the firefly luciferase gene. A construct pHSPT70L contains the promoter and exon regions of the human HSP70 gene (−188 to +150) upstream from the luciferase gene. The DNA segment of the HSP70 gene was derived from a HSP70 subclone LSN. A promoterless construct, pLLI, was constructed by self-ligating a linearized pPHOLS at the unique HindIII site, both ends of which had been filled in before ligation.

**Transient expression analysis.** K1735 mouse melanoma cells were cultivated in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum and were transfected essentially as described previously. K1735 cells, seeded in a 10-cm diameter dish, were fed with fresh medium 2 hours before addition of each pHHOgp construct (20 μg DNA) together with or without 5 μg of pSV2/L, harboring the SV40 early-region promoter sequence linked to the luciferase gene. After glycerol treatment, cells were incubated for 20 hours and then treated for 3 hours at 42°C or 37°C. For detection of the reporter gene transcripts, total RNA (20 μg) prepared from transfected cells was analyzed for the expression of gpt transcripts or both gpt and luciferase transcripts by S1 nuclease mapping, as described. The S1 probes used are indicated in the figure legends. Radioactivity of the protected products was visualized by exposing to an x-ray film.

YN-1-0-A cells were transfected by the diethyl aminoethyl (DEAE)-dextran method as described previously. Cells (5 × 10⁴ to 1 × 10⁵) were incubated in a 15-mL polystyrene tube for 30 minutes in 1.0 mL of IMDM containing 500 μg/mL of DEAE-dextran, 5 μg of a test promoter-luciferase fusion gene, and 1 μg of β-galactosidase expression vector, pCH110 (Pharmacia, Uppsala, Sweden), as an internal control. The fusion genes used were pPHOLS, pPHOL14, pPHOL15, and pPHOL70L (see Fig 7). After transfection, cells in each tube were collected, resuspended with fresh IMDM containing 1% FCS, and transferred to 12-well plates (1 × 10⁶ cells in 1.5 mL medium per well). After 24 hours of incubation, cells were treated for 5 hours with hemin (5 μmol/L) or Cd (5 μmol/L) at either 37°C or 42°C. Cellular extracts were then prepared for assays of luciferase and β-galactosidase activities. The luciferase activity was normalized by dividing with each β-galactosidase activity to calculate the relative luciferase activity and shown as a ratio to the value obtained with each construct (untreated at 37°C).

**Northern blot analysis.** YN-1-0-A cells were cultivated in serum-free medium (1 × 10⁶ cells/mL) for 3 or 3.5 hours under the conditions shown in Fig 6. Total cellular RNA was prepared from each dish (1 × 10⁵ cells) according to the method of Razaoli B (Cinnam/Biotec Laboratories Inc, Houston, TX) and subjected to Northern blot analysis as described. The RNAs blotted on the filters were fixed by brief exposure to UV irradiation and were hybridized with 32P-labeled cDNA probes. The hybridization probes used for heme oxygenase-1 and HSP70 mRNAs were the Xho U2b region (positions −921 to 246) derived from the human heme oxygenase-1 cDNA, pHHO1, and the HindIII/BamHI fragment derived from a subclone of the human HSP70 gene, pHZ2.3, respectively. The human β-actin probe was the Sma I/Sac I fragment (124/1050), derived from a full-length cDNA provided by Tokuo Yamamoto (Gene Research Center, Tohoku University, Sendai, Japan). The nucleotide residues of a β-actin cDNA were numbered according to the published sequence. All these probes were labeled with α-32PdCTP by the random priming method. Hybridization signals were detected by autoradiography.

**RESULTS**

Heme oxygenase-1 is not induced by heat shock in human alveolar macrophages. HHO-HSE is located between...
Fig 1. The cis-acting elements of the 5'-flanking region of human heme oxygenase-1 gene. (A) Nucleotide sequence of the 5'-flanking region of human heme oxygenase-1 gene. The nucleotide sequence of the coding strand is displayed in 5' to 3' orientation. The transcription initiation site (nucleotide residue 1) is marked with an asterisk. HSE represents the sequence consisting of four contiguous 5-bp units underlined or a classic 14-bp palindromic sequence overlaid (see B). Also underlined are the purine-rich region, the polyId(GT) region, MTE responsible for myelomonocytic cell-specific transcription by TPA, the USF-binding region, and a TATA-like sequence.

Comparison of HSEs. The HSE of the human heme oxygenase-1 gene (HHO-HSE) is compared with the functional HSE (positions -290 to -271) of the rat heme oxygenase-1 gene15,16 and the HSE (positions -115 to -91) of the human HSP70 gene. Only matches with the HSE consensus sequence (5'-NGAAN-3') are indicated in capital letters. Solid arrows over the sequences indicate a 5-bp unit with full match to the HSE consensus sequence and its orientation. Dotted arrows over the sequence indicate the 5-bp unit with mismatch. Underlining indicates the sequence matching to the classic 14-bp consensus of HSE.18

-384 and -365 (Fig 1A) and consists of four 5-bp inverted repeats, of which three are perfect matches and one is an imperfect match to the consensus (5'-NGAAN-3'; Fig 1B). For comparison, the human HSP70 HSE, consisting of five pentamers, is also shown. Each pentamer of which was shown to be bound by heat shock factor (HSF). A classic HSE of 14 bp is common to both human and rat heme oxygenase-1 genes and the human HSP70 gene. To clarify whether human heme oxygenase-1 is induced by heat shock, we analyzed the effects of heat shock (treatment at 42°C) on the expression of heme oxygenase-1 protein in primary culture of alveolar macrophages. The relative amounts of heme oxygenase-1 protein were measured by Western blot analysis. As shown in Fig 2A, heat shock rather reduced the amounts of heme oxygenase-1 protein, whereas HSP70 protein was apparently induced. It should be noted that the same blot used for heme oxygenase-1 was reused for detecting HSP70 protein.

We next performed a more comprehensive series of heat shock studies using an erythroid cell line, YN-1-0-A. Preliminary experiments established that HSP70 mRNA is more efficiently induced at 42°C as compared with the induction at 40°C or 44°C (data not shown). We thus analyzed the effects of heat shock (42°C) on the levels of heme oxygenase-1 protein and the activity of heme oxygenase. As a positive control, cells were treated with hemin, because it is
well known that hemin remarkably induces heme oxygenase-1 in human cells. Heme oxygenase-1 protein was increased in the hemin-treated YN-1-0-A cells, but no increase was detected in the cells treated at 42°C (Fig 2B). Consistent with this observation, there were no detectable differences in the activity of heme oxygenase between the untreated cells and the cells treated by heat shock (42°C), whereas hemin induced the activity twofold (data not shown). In contrast, HSP70 protein was induced in the cells treated with hemin or by heat shock. It has been also confirmed that heme oxygenase-1 protein was not induced at 40°C or 44°C in both YN-1-0-A cells and HeLa human cervical cancer cells (data not shown).

**Binding of a heat shock factor to HHO-HSE.** As a first step to assess the function of HHO-HSE, we performed gel mobility-shift assays using whole-cell extracts of YN-1-0-A cells treated for 0.5 hours under the indicated conditions (Fig 3). It has been reported that treatment of HeLa cells at 42°C for 0.5 hours increases the DNA-binding activity of HSF to the maximal levels. The fragments containing HHO-HSE, the *Dde I/Str I* fragment (−405/−344; designated as f42) and the *Dde I/Ban II* fragment (−405/−315; f41), were bound by a nuclear protein, possibly HSF, because the DNA-binding activity was remarkably increased in heat-treated cells. A 50-fold molar excess of a specific competitor competed for the complex formation with the labeled probe, whereas an unspecific competitor did not (data not shown). Similar binding assays were performed by using an HSP70 probe, the *Ava II/Tag I* fragment of 69 bp, containing the five 5-bp motifs, showing that the DNA-binding activity of HSF was activated under the conditions used (treated at 42°C for 0.5 hours; lanes 9, 10, and 13). These results suggest that HSF is able to bind to HHO-HSE at least in vitro, which is consistent in part with a previous report by other investigators. Interestingly, hemin treatment caused a small increase in the formation of the DNA/HSF complexes (lanes 3, 7, and 11), whereas Cd treatment caused no detectable increase (lanes 4, 8, and 12). We also obtained similar results using nuclear extracts of the heat-shocked cells (data not shown).

**Functional analysis of the human heme oxygenase-1 gene promoter.** We then analyzed the promoter function of the human heme oxygenase-1 gene by transient expression assay in K1735 mouse amelanotic melanoma cells, because this cell line is of higher efficiency in transient expression of the introduced fusion genes compared with HeLa and YN-1-0-A human cell lines, and thus the reporter gene transcripts could be detected by S1 mapping analysis. A parent construct pHHOgpt3 contains the 991-bp promoter region upstream from the gpt reporter gene, and pHHOgpt3Δ4 lacks the polycl(GT) region of about 110 bp (Fig 4A). The basal expression of pHHOgpt3 containing HHO-HSE was detectable but was not increased by the treatment with Cd or hemin or by heat shock (Fig 4B). The lack of induction by Cd or hemin is consistent with our recent report that the Cd-responsive element is located 4 kb upstream from the transcriptional initiation site but the hemin-mediated induction of a reporter gene is not detectable (see Fig 7). In contrast, the expression of pHHOgpt40, containing a synthetic HHO-HSE (−383 to −368), was noticeably increased upon heat shock, although its basal expression was undetectable. The amounts of undigested probe were sometimes increased when the expression of a fusion gene was remarkably induced, as seen in lane 8 of Fig 4B. Such an increase in the undigested probe may be due to the increased transcription of a fusion gene, initiated from the aberrant initiation sites under thermal stress. The
increased amounts of undigested probe were also observed when heat shock remarkably increased the reporter gpt expression under the control of the rat heme oxygenase-1 promoter. The internal deletion of the poly[d(GT)] region did not confer the inducibility of the reporter gene by heat shock (lanes 9 and 10).

**Functional analysis of HHO-HSE.** To further explore the function of HHO-HSE, we examined the effects of various internal deletions located downstream from HHO-HSE on the heat-mediated induction of fusion gene transcripts (Fig 5). In this series of transient expression assays, pSV2/L containing the luciferase gene linked to the SV40 promoter was included as an internal control. Under basal conditions, the gpt transcripts of any fusion genes were not detectable, whereas the luciferase transcripts were detected in all samples. After heat shock, the gpt transcripts were detectable in the cells transfected with the fusion genes containing a deletion downstream from HHO-HSE. For example, expression of pHHOgpt41 and pHHOgpt42, carrying the f41 and f42 fragments used in gel mobility-shift assays, was remarkably induced by heat shock. Furthermore, the Alu I/BstN1 fragment (f45) of 24 bp containing HHO-HSE, carried by pHHOgpt45, was sufficient to confer the maximum heat-mediated induction. The magnitude of activation was smaller with pHHOgpt41 than with pHHOgpt42 by fourfold, although both f41 and f42 fragments were similarly bound by heat shock factor in vitro (see Fig 3). Thus, the presence of the additional 28-bp sequence carried by pHHOgpt41, the region between the Sty I and Ban II sites, reduced the heat-mediated transcriptional activation of the human heme oxygenase-1 gene.

**Effects of heat shock on the hemin- and Cd-mediated induction of the heme oxygenase-1 mRNA.** To explore the regulation of the heme oxygenase-1 gene under thermal stress, we analyzed the effects of heat shock on the hemin- or Cd-mediated induction of heme oxygenase-1 and HSP70 mRNAs in YN-1-0-A cells (Fig 6). It has been established that Cd acts at the transcriptional level to induce the human heme oxygenase-1 gene and increases the amounts of heme oxygenase-1 protein. Treatment with either hemin or Cd remarkably increased the levels of both heme oxygenase-1 and HSP70 mRNAs (lanes 1, 9, and 10), whereas heat shock of 3.5 hours induced only HSP70 mRNA (lane 5). The 0.5 hours of exposure to heat shock did not induce HSP70 mRNA expression (lane 4). The additional bands seen with a heme oxygenase-1 probe may represent the aberrant heme oxygenase-1 gene transcripts, as previously observed. On the other hand, an additional band seen with an HSP70 probe may represent the mRNA related to HSP70. Interestingly, heat shock of 3.5 hours completely inhibited the induction...
REGULATION OF HUMAN HEME OXYGENASE-1

Fig 4. Functional analysis of the human heme oxygenase-1 gene promoter. (A) Schematic representation of chimeric fusion genes. Negative numbers at the top of the panel indicate the nucleotide residues from the transcription initiation site of the human heme oxygenase-1 gene. Arrows indicate the direction of transcription. An open box represents a part of exon 1 of the human heme oxygenase-1 gene (numbered as 1) and the reporter gpt gene. (II), (III), and (III) indicate HHO-HSE, the purine-rich region, and the poly[dlGT] region, respectively. The sloping line indicates an internal deletion. pHHOgpt40 contains a synthetic HHO-HSE at the 5' end of pHHOgpt4. (B) S1 mapping analysis for the reporter gene transcripts. K1735 cells were transfected with the indicated fusion genes and were then treated with Cd or hemin or by heat shock (42°C). The S1 probe used is shown in Fig 5B. The protected fragments of 236 nucleotides from the digestion with S1 nuclease are indicated by an arrow (HHOgpt). An arrowhead indicates undigested S1 probe. Lanes indicated with (-) contain the protected fragments by RNA of the untreated transfected cells. The size markers were the end-labeled pUC8 DNA digested with Hpa II (lane M).

of heme oxygenase-1 mRNA caused by hemin (lane 2) or Cd (lane 7), whereas a short exposure of 0.5 hours caused only little or no reduction in the magnitude of induction (lanes 3 and 8). Similar inhibitory effects of heat shock on the induction of heme oxygenase-1 mRNA were also observed in HeLa cells (data not shown). In contrast, expression of HSP70 mRNA was always induced after heat shock of 3.5 hours, even in combination with hemin (lane 2) or Cd (lane 7).

Effects of heat shock on the cadmium-mediated increase of the heme oxygenase-1 promoter activity. To explore the differences in the promoter function between the heme oxygenase-1 and HSP70 genes, transient expression assays were performed in YN-I-0-A cells (Fig 7). The luciferase gene was used as a reporter, because the sensitive assay method of luciferase activity allowed us to detect the reporter gene expression in YN-I-0-A cells (data not shown). In contrast, expression of HSP70 mRNA was always induced after heat shock of 3.5 hours, even in combination with hemin (lane 2) or Cd (lane 7).

DISCUSSION

Human heme oxygenase-1 is a stress protein and its gene promoter region contains various potential cis-acting elements, including HHO-HSE, which are bound by nuclear
proteins in vitro, In the present study, we have provided several lines of evidence that human heme oxygenase-1 is not an HSP in an erythroid cell line, YN-1-0-A. The amounts of heme oxygenase-1 protein were not increased by heat shock in YN-1-0-A cells (Fig 2). The 5'-flanking region of the human heme oxygenase-1 gene containing HHO-HSE is not able to confer the heat-mediated inducibility on the reporter gene, but the synthetic HHO-HSE or the deletion of the sequence located downstream from HHO-HSE could lead to confer the heat-mediated increase in the reporter gene expression (Figs 4 and 5). Consistent with the latter observation, gel mobility shift assays indicate that HSF binds to the region containing HHO-HSE (Fig 3). All these results suggest that HHO-HSE is potentially functional, but its function is repressed in vivo. However, the repression of the function of HHO-HSE is not always complete, because we and other groups observed a small but noticeable increase in the levels of heme oxygenase-1 mRNA in some cell lines upon heat shock. In contrast, there were no reports showing the induction of heme oxygenase activity in human cell lines by heat shock. Rather, it has been reported that heme oxygenase activity is not increased by heat shock. Here, using the primary culture of human alveolar macrophages, we also showed that the level of heme oxygenase-1 protein was reduced by heat shock, whereas HSP70 was noticeably induced (Fig 2A). These results suggest that heme oxygenase-1 is not an HSP in human alveolar macrophages.

There were apparent differences in the induction of the DNA-binding activity of HSF in YN-1-0-A cells among the conditions used (Fig 3). Heat shock is the most efficient stress that rapidly increases the DNA-binding activity of HSF, although we were unable to detect the DNA-binding activity of HSF in the cells treated with Cd for 0.5 hours (Fig 3). The latter observation is consistent in part with the report that the maximal activation in the DNA-binding activity of HSF was obtained after 2 hours treatment with Cd. In this context, transient expression assays showed that the expression of both pHOL15 and pHSP70L was increased by Cd (Fig 7), although the Cd-mediated activation of the heme oxygenase-1 gene promoter is not mediated by HHO-HSE. Thus, the mechanism for accumulation of heme oxygenase-1 mRNA caused by Cd is different from that for HSP70 mRNA in YN-1-0-A cells. On the other hand, the small increase in the DNA-binding activity of HSF was seen in the hemin-treated cells, which is consistent in part with the reports that hemin induces HSP70 gene expression by activating HSF. However, hemin treatment exerted no noticeable effects on the expression of a reporter gene under the control of either the heme oxygenase-1 or HSP70 promoter.

It is unlikely that the lack of heat-mediated inducibility of the heme oxygenase-1 gene is due to the relatively far upstream location of HHO-HSE (positions -384 to -365), because the internal deletion of the poly[d(GT)] region did

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**Fig 5. Effects of the internal deletion on the function of HHO-HSE.** (A) Nucleotide sequence of HHO-HSE and its adjacent regions. Fragments (f41–f45) shown were ligated to the HindIII site of pHHOgpt4. (B) Schematic representation of chimeric fusion genes. The structure of pSV2/L is also shown. Asterisks indicate the radiolabeled ends of the S1 probes used to detect gpt and luciferase transcripts. (C) S1 mapping analysis for the reporter gene transcripts. K1735 cells were cotransfected with each fusion gene shown in (B) and pSV2/L. A solid and an open arrowhead indicate the undigested S1 probe for the HHO-gpt (356 nucleotides) and the luciferase reporter gene (about 446 nucleotides) yielding the protected fragments of 236 nucleotides for gpt transcripts (HHO-gpt) and those of about 144 nucleotides for luciferase transcripts (internal control).
not affect the expression of the reporter gene upon heat shock (Fig 4B). We rather suggest that the region located downstream from HHO-HSE, such as the purine-rich region, may act as a silencer (Fig 5). It should be noted that heat shock abolished the accumulation of heme oxygenase-1 mRNA in YN-1-0-A cells treated with hemin or Cd (Fig 6), whereas the combination of heat shock with hemin or Cd did induce HSP70 mRNA. Consistent with these observations, transient expression assays showed that the Cd-mediated increase in the expression of pHHOL15 was not detected at 42°C, whereas the expression of pHS70L was higher at 42°C than that at 37°C (Fig 7). These results indicate that the regulation of heme oxygenase-1 gene expression is different from that of the HSP70 gene in YN-1-0-A cells under heat shock conditions, supporting that human heme oxygenase-1 is not an HSP in YN-1-0-A cells.

Rat heme oxygenase-1 was established as an HSP15,16 and its gene promoter contains the functional HSE (−290 to −271) and an HSE-like sequence (−221 to −212) consisting of an inverted repeat of 5′-NGAAN-3′, as shown in Fig 1B. The latter HSE-like sequence is by itself unable to confer heat-mediated induction but acts synergistically with the functional HSE.16 To further explore the function of the putative silencer sequence for HHO-HSE, we analyzed the effects of insertion of the purine-rich region on the heat-mediated inducibility of the fusion genes under the control of the rat heme oxygenase-1 gene promoter. The purine-rich sequence was inserted at the two sites downstream from the HSE of the rat heme oxygenase-1 gene. However, no apparent effects on the amounts of the transcripts were detected (data not shown). In addition, we analyzed the effects of insertion of either the purine-rich sequence or the poly[d(GT)] element on the function of the HSP70 HSE. Transient expression assays in YN-1-0-A cells showed that the expression of the HSP70-luciferase fusion genes containing each DNA fragment downstream from HSP70 HSE was not increased at 42°C, whereas the expression of a wild-type construct was remarkably increased at 42°C (data not shown). These results suggest that insertion of any sequence downstream from HSP70 HSE may inhibit its function upon heat shock. All these results suggest that the silencing effect of the purine-rich sequence is specific for the human heme.
Induction of relative luciferase activity

Heme oxygenase-1
-232 +1
pHHOL5
-4.0 kb -300 +1
pHHOL14
-4.5 kb -4.0 kb
pHHOL15
CdRE
-37°C
-42°C
37°C
42°C

HSP 70
-188 +1
pHSP70L
-37°C
42°C
37°C
42°C

Promoterless
-Luc
pLL1

Control Hemin Cadmium 42°C

Fig 7. Functional analysis of HHO-HSE and HSP70 HSE in YN-1-0-A cells. Each fusion gene contains the 5'-flanking region of the human heme oxygenase-1 or HSP70 gene upstream from the luciferase gene. The CdRE represents the Cd-responsive element located about 4 kb upstream from the transcriptional initiation site. YN-1-0-A cells were cotransfected with each fusion gene and β-galactosidase expression vector and then incubated at 37°C or 42°C under the conditions indicated. The magnitude of induction is shown as a ratio to the relative luciferase activity of untreated cells (control) transfected with each construct. The data shown are the mean ± SD of three independent experiments.

Oxygenase-1 gene promoter or the position of the sequence relative to HHO-HSE is crucial for transcriptional repression.

Heme oxygenase-1 was suggested to constitute a defense system against oxidative stress, because biliverdin or bilirubin produced locally may work as a physiologic antioxidant. However, it is not easy to evaluate whether induction of heme oxygenase-1 is beneficial or harmful for the host, because the heme breakdown products, biliverdin/bilirubin, carbon monoxide, and iron, are considered metabolic double-edged swords. For example, carbon monoxide is a noxious gas but was shown to possess certain physiologic functions similar to those of nitric oxide. Iron, an essential element for living organism, may be involved in the production of hydroxyl radicals that are extremely reactive with biologic components. Thus, if the heme oxygenase-1 gene is induced by heat shock, local concentrations of these products are easily increased under the conditions such as high fever and exercise, which may result in the elevation of body temperature and, in turn, induce heme oxygenase-1. Indeed, it was reported that hyperthermia increased the amounts of heme oxygenase-1 protein and mRNA levels in the rat brain. It is therefore conceivable that some species, including humans, may acquire a regulatory system that prevents the noxious induction of heme oxygenase-1 by heat shock, whereas the property of heme oxygenase-1 as a stress protein is well conserved. Such a novel regulatory mechanism is of vital importance, because heme oxygenase-1 plays an important role in differentiation of bone marrow cells. The present study will provide a new insight into the physiologic significance of the induction of heme oxygenase-1 by stress.

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