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Involvement of Heat Shock Elements and Basal Transcription Elements in the Differential Induction of the 70-kDa Heat Shock Protein and Its Cognate by Cadmium Chloride in 9L Rat Brain Tumor Cells

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Abstract Exposure of 9L rat brain tumor cells to 40–100 μM $CdCl_2$ for 2 h leads to an induction of a wide spectrum of heat shock proteins (HSPs). We have demonstrated that induction of the 70-kDa HSP (HSP70) and enhanced expression of its cognate (HSC70) by cadmium are concentration dependent and that the induction kinetics of these HSP70s are different. The increased synthesis of the HSP70s is accompanied by the increase in *hsp70* and *hsc70* mRNA levels, indicative of transcriptional regulation of the heat shock genes. Electrophoretic mobility shift assay (EMSA) using probes encompassing heat shock element (HSE), TATA, GC, and CCAAT boxes derived from the promoter regions of the heat shock genes shows distinguished binding patterns between *hsp70* and *hsc70* genes in both control and cadmium-treated cells. The results indicate that, in addition to the HSEs, the basal transcription elements are important in the regulation of the heat shock genes. The binding patterns of the corresponding transcription factors of these elements are examined by EMSA by using extended promoter fragments from respective heat shock genes with sequential addition of excess oligonucleotides encompassing individual transcription elements. Taken together, our results show that the differential induction of *hsp70* and *hsc70* involves multiple transcription factors that interact with HSE, TATA, GC, and CCAAT boxes. J. Cell. Biochem. 71:21–35, 1998.

Key words: heat shock protein; heat shock genes; heat shock element; heat shock factor; basal transcription elements

Cadmium is a systemic poison affecting many cellular functions and its effects are cell-type specific and dose dependent [Beyersmann and Hechtenberg, 1997]. In most systems tested, cadmium enhances the expression of genes coding for the synthesis of protective molecules including glutathione [Bannai et al., 1991; Chin and Templeton, 1993; Li et al., 1993] and metallothionein [Koropatnick and Leibbrandt, 1995]. At very low concentrations (e.g., micromolar and down to nanomolar ranges) and with transient exposure (1 or 2 h), cadmium stimulates DNA synthesis and cell growth in cultured mammalian cells [Zglinicki et al., 1992]. Under such conditions, cadmium stimulates the expression of several cellular proto-oncogenes that are generally associated with cell proliferation [Epner and Herschman, 1991; Matsuoka and Call, 1995; Abshire et al., 1996; Hechtenberg et al., 1996]. At elevated concentrations and prolonged exposure, cadmium is highly cytotoxic and inhibits all levels of genetic information transfer [Cohen et al., 1991]. Moreover, in cells treated with cytotoxic levels of cadmium, the metal ion is often found to induce the synthesis of a set of stress proteins that are commonly referred to as the heat shock proteins (HSPs) [Lai et al., 1993; Ovelgonne et al., 1995].

HSPs are a small set of proteins induced in cells subjected to supraoptimal temperature and other physiological stresses [Lindquist and Craig, 1988; Nover, 1991]. The majority of these proteins are demonstrated to function as molecular chaperones in both normal and stressed cells [Georgopoulos and Welch, 1993; Rassow and Pfanner, 1995]. The most abundant and

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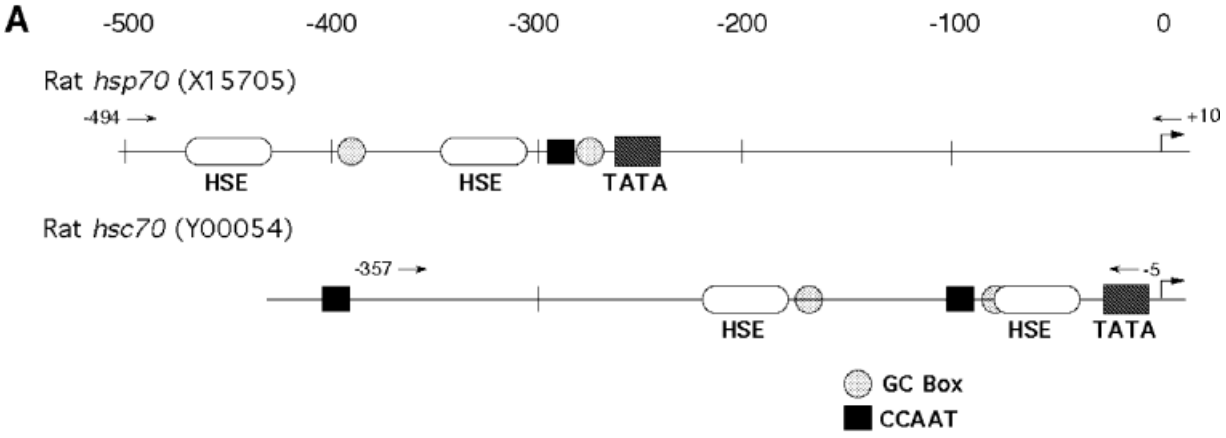
best characterized HSPs are a family of proteins with molecular mass around 70-kDa (referred to as the HSP70s). In mammalian cells, the *hsp70* gene family includes stress-inducible *hsp70* gene, constitutively expressed cognate *hsc70* gene, and constitutively expressed glucose-regulated *grp78* (BiP) and *grp75* genes [Welch, 1991]. Both HSP70 and HSC70 are cytosolic/nucleus residing [Hightower and White, 1981; Lee et al., 1992; Wang et al., 1998]. These two HSP70s possess weak ATPase activity that is stimulated with peptide binding [Flynn et al., 1989] and can dissociate some protein aggregates coupled with ATP hydrolysis [Pelham, 1990]. HSP70 and HSC70 are highly related (>90% sequence identity) [Lindquist and Craig, 1988] and are often suggested to have similar physiological functions [Brown et al., 1993; Rassow et al., 1995; Freeman and Morimoto, 1996]. Despite the high level of similarities between HSP70 and HSC70, the expression of these proteins in control and stimulated cells is distinguishably different. In general, HSP70 is rarely expressed under normal conditions and is highly stress inducible, whereas HSC70 is constitutively expressed but is only slightly stress inducible [Pelham, 1986; Wang et al., 1998]. However, the detail induction kinetics of HSP70 and HSC70 have still not been established in most of the experimental systems.

The activation of heat shock gene transcription, including that of *hsp70* and *hsc70*, during the stress response is mediated mainly by heat shock transcription factor-1 (HSF1), which binds heat shock elements (HSEs) in the promoter region and transactivates the heat shock genes [Baler et al., 1993; Morimoto, 1993; Sarge et al., 1993]. Although the importance of HSF1 in the regulation of mammalian *hsp*/*hsc70* expression is well established, several lines of evidence demonstrate that activation of HSF1 alone is not sufficient for the induction of the *hsp*/*hsc70* genes, indicative of the existence of additional regulatory factors [Liu et al., 1993; Morimoto, 1993; Mathur et al., 1994]. In fact, with regard to the HSEs, a dual control mechanism for the regulation of the heat shock genes has been proposed: a positive control mediated by the HSF1 and a negative control mediated by the constitutive heat shock element-binding factor (CHBF) [Liu et al., 1993], which has been identified as the Ku autoantigen [Yang et al., 1996]. Moreover, it has been shown that the transcription of heat shock gene may be controlled independently of the HSFs by the Ha-Ras signaling pathway [Engelberg et al., 1994]. It is now clear that the promoters of these genes are regulated by the complex interplay of several *cis*-elements and protein factors binding to these sites. For instance, in addition to the HSEs, regulatory elements that may interact with SRF-, SP1-, TFIID-, CTF-, and ATF-like factors have been characterized in the human *hsp70* promoter [Wu et al., 1986; Morgan et al., 1986; Schiller et al., 1988; Williams et al., 1989; Lum et al., 1990; Williams and Morimoto, 1990]. Specifically, it has been demonstrated that the basal expression of human *hsp70* represents the contribution of multiple elements, including the CCAAT box, the GC box (Sp1 site), the TATA box, and an ATF- or AP1-like element [Wu et al., 1986; Greene et al., 1987; Williams et al., 1989] and that maximal stress-induced transcription from the human *hsp70* promoter requires interactions with the basal promoter elements [Williams and Morimoto, 1990]. Sequence analysis of the promoter regions of the rat *hsp70* and *hsc70* shows that, in addition to the HSE, there are also other putative regulatory elements including TATA, GC, and CCAAT boxes in these promoters (Fig. 1). However, the functionality and cooperativity of these regulatory elements leading to the differential expressions of *hsp70* and *hsc70* under stress have never been characterized.

We previously reported that treatment of 9L rat brain tumor (RBT) cells with cadmium results in the induction of almost all of the major stress proteins and that rat HSP70 and HSC70 are differentially induced by cadmium [Lai et al., 1993]. In the present study, we first established the differential expression kinetics of rat HSP70 and HSC70 by treating the 9L RBT cells with various dose of cadmium. Subsequently, we used this system to investigate the promoter activity of these two genes in cells under cadmium stress. Our results demonstrate that the differential induction of rat *hsp70* and *hsc70* by cadmium is mediated by the expression of different binding activities toward the HSEs located in the promoter regions of *hsp70* and *hsc70*. Moreover, the process may also rely on the binding activities of transcription factors in addition to HSF that would interact with basal



B Synthesized Oligonucleotides:

hsp70

HSE: 5'-CGGAGAAGCTCTAACAGACCCGAAACTGCTGGA-3' (-349 — -317)
CCAAT: 5'-CGCTGATTGGCCCATGGG-3' (-298— -281)
GC box: 5'-GGGTGGGCGGGGCCGGAG-3' (-278— -261)
TATA: 5'-GGAGCCTCCTTAAAGGGGC-3' (-262— -244)

hsc70

HSE: 5'-GCGAGCGTGAAAGTTCCAGAACGCTGCGGTCGTGCGG-3' (-220— -184)
CCAAT: 5'-GCCGTCCATTGGCTAAGG-3' (-103— -86)
GC box: 5'-CGGAGGGGCGGAGTGGAGGCG-3' (-177— -158)
TATA: 5'-ACAGGGTATAAGAGACAGTC-3' (-37— -18)

Fig. 1. Schematic drawings of the promoter regions of rat *hsp70* and *hsc70* genes and oligonucleotide sequences synthesized. The DNA sequences of the promoter regions were obtained from the gene bank and the respective sequences from +1 to −501 (*hsp70*) and +1 to −422 (*hsc70*) are presented. **A:** The boxes represent the approximate locations of the regulatory elements of interest. Arrows represent oligonucleotides synthesized and are used as primers to obtain the "extended promoter fragments" by PCR. **B:** Also shown are oligonucleotide sequences, representing the basal transcription elements, synthesized and used as EMSA probes.

promoter elements including the TATA, GC, and CCAAT boxes.

MATERIALS AND METHODS

Materials

Cultureware was obtained from Corning Glass Works (Corning, NY) and culture medium components were purchased from Gibco Laboratories (Grand Island, NY). [^{35}S]methionine (specific activity > 800 Ci/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), and [γ-^{32}P]ATP (5,000 Ci/mmol) were purchased from Amersham (Buckinghamshire, England). Bio-Thermal DNA polymerase was purchased from K.S.T. (Brea, CA). Synthetic oligonucleotides were ordered from Gibco-BRL (Gaitherburg, MD). Chemicals for electrophoresis were obtained from Bio-Rad (Richmond, CA). Other chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Cell Culture and Drug Treatment

The 9L RBT cells, derived from rat gliosarcoma, were a gift from Dr. M.L. Rosenblum (University of California at San Francisco) and were maintained in Eagle's Minimum Essential Medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin [Weizaecker et al., 1981]. Prior to each experiment, stock cells were plated in 25-cm^2 flasks or six-well plates at a density of 4–6 $\times$ 10^4 cells/cm^2. Exponentially growing cells at 80–90% confluency were used. Cadmium chloride was dissolved in water at a concentration of 100 mM and was added to the culture medium to the desired concentrations for treat-

ment. The cells were treated at 37°C for 2 h and allowed to recover for different durations as specified in the following sections and figure captions.

Metabolic Labeling and Gel Electrophoresis

Synthesis of stress proteins in the cadmium-treated cells was monitored by [^{35}S]methionine labeling. The 9L RBT cells were treated with 40–100 μM $CdCl_2$ for 2 h and allowed to recover under normal growing conditions for up to 24 h. After treatment and recovery, the cells were labeled with [^{35}S]methionine (20 μCi/ml) for 1 h before being harvested. The cells were then washed with ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4), lysed with sample buffer, and subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli, 1970]. The samples were applied to the gels on the basis of same amounts of cell lysate. After electrophoresis, the gels were stained, destained, and processed for autoradiography by using Fuji RX x-ray film as described elsewhere [Lee et al., 1991]. Bands of interest in autoradiographs were quantified by densitometric scanning (Molecular Dynamics, Sunnyvale, CA) and processed for presentation by Adobe Photoshop 3.0 (Adobe, CA).

Polymerase Chain Reaction (PCR)

Genomic DNA was isolated from the 9L RBT cells as described elsewhere [Gross-Bellard et al., 1973]. The PCR primers for producing the rat *hsp70* and *hsc70* probes for Northern hybridization were, with respect to the coding sequences, 5′-TCGAGGAGGTGGATTAGAG-3′ (2411 to 2429, sense) and 5′-ACGCAAAGAACATGCAAC-3′ (2628 to 2611, antisense) for *hsp70* and 5′-GCTGACGGGTCACGCATG-3′ (2980 to 2997, sense) and 5′-TTTCCGCCCGCTTCACTGT-3′ (3267 to 3247, antisense) for *hsc70*. However, the PCR primers for generating the "extended promoter fragments" of rat *hsp70* and *hsc70* for EMSAs were, with respect to the genomic sequences, 5′-GCAACACTGTCACAACCGGA-3′ (−494 to −475, sense) and 5′-TACTGGTCATTCTTAGCGG-3′ (+10 to −9, antisense) for *hsp70* promoter and 5′-GCTGACGGGTCACGCATG-3′ (−357 to −340, sense) and 5′-TTTCCGCCCGCTTCACTGT-3′ (−5 to −23, antisense) for *hsc70* promoter. PCR reactions were performed in a volume of 50 μl in a thermal cycler (Hybaid, Teddington, UK). For each reaction, 50 pmol of oligonucleotide primer and 1 μg DNA template were incubated with 1 U of Bio-Thermal polymerase and 200 μM dNTPs according to the method of Saiki et al. [1988]. The following amplification protocol was used. The first cycle consisted of 3 min denatruation at 94°C. The following cycles consisted of 1 min denaturation at 95°C, 30 sec annealing at 50°C, and 30 sec extension at 72°C. The reaction was allowed to proceed for 35 cycles. The amplified products were electrophoresed on 1.5% agarose gels. The gels were stained with ethidium bromide, and the DNA fragments with expected length were excised and purified further by a commercial kit (Genie Prep, Ambion, Austin, TX).

RNA Isolation and Northern Hybridization

Total RNA was isolated from 9L RBT cells by using an RNA isolation kit (TRIZOL reagent, Gibco BRL) according to the protocol specified by the manufacturer. The RNA samples were subsequently fractionated on 1% agarose gels. After electrophoresis, the gels was incubated in 0.05 N NaOH for 30 min and washed with 2× standard saline citrate (SSC) buffer. The RNA samples were then electrotransferred onto nylon membranes (Hybond-N, Amersham) in 10× SSC buffer for at least 12 h. The membranes were then dried, and the RNA samples were fixed onto the membranes by using an ultraviolet crosslinker (Stratagene, La Jolla, CA). The membranes were equilibrated in the hybridization solution (6× SSC, 0.5% SDS, 5× Denhardt's solution, 100 μg/ml salmon sperm DNA, and 50% formamide) for 12 h at 42°C. The detection for *hsp70* and *hsc70* mRNAs were performed by using the respective PCR products that were labeled with [α-^{32}P]dCTP by the Rediprime DNA labeling system (Amersham), and the hybridization was carried out in the hybridization solution at 42°C for 15 h. Subsequently, the membranes were washed with 1× SSC containing 0.1% SDS at room temperature followed by another wash in 1× SSC containing 0.1% SDS at 65°C for 10 min. The membranes were then dried and processed for autoradiography. Bands on autoradiograms were quantified by densitometric scanning using 18S rRNA as the internal controls.

Nuclear Extract Preparation

Nuclear extracts were prepared from 9L RBT cells by a rapid fractionation protocol [Hen-

nighausen and Lubon, 1987]. About 10^7 cells were trypsinized, collected by centrifugation at 100*g* for 8 min at 4°C, washed once with PBS, and centrifuged as above. The pellet was resuspended in 0.5 ml of nuclear extraction buffer (10 mM Hepes-KOH pH 7.9, 0.5% Triton X-100, 0.5 M sucrose, 0.1 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and then homogenized by three strokes with a Dounce grinder. The samples were briefly centrifuged at 10,000*g*, and the resulting pellet was lysed in 0.5 ml nuclear extraction buffer supplemented with 0.5 M NaCl and 5% glycerol at 4°C for 30 min. The samples were then centrifuged at 14,000*g* for 20 min, and the supernatant fractions were collected. The nuclear extracts obtained were dialyzed for 3–4 h against at least 50 volumes of dialysis buffer (10 mM Hepes-KOH, pH 7.9, 17% glycerol, 0.1 mM EDTA, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and kept frozen at −70°C.

Electrophoretic Mobility Shift Assay

Synthetic oligonucleotides were used for the detection of protein factors that bind to specific regulatory elements including HSE, TATA, GC, and CCAAT boxes. The extended promoter fragments of *hsp70* and *hsc70* generated by PCR were used for the study of the cooperative binding of the nuclear factors in the cadmium-treated cells. The PCR products and the annealed synthetic oligonucleotides were endlabeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. In the binding reaction mixture, 2 µg of nuclear extract were incubated in a 15-µl reaction containing 10 mM Hepes-KOH, pH 7.9, 5 mM $MgCl_2$, 4 mM Tris-HCl, 12% glycerol, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 300 µg BSA/ml, and 20 µg poly(dI-dC). For competition assay, 100× excess unlabeled probes were added concurrently in the reaction mixture. Free DNA and DNA–protein complexes were resolved on 4% nondenaturing polyacrylamide gels in Tris/Borate/EDTA buffer. After electrophoresis, the gels were dried and processed for autoradiography.

RESULTS

Treatment With Cadmium Induces a Wide Spectrum of Stress Proteins in 9L RBT Cells

Induction of stress proteins by chemical stressors is dependent on the concentrations applied and on treatment protocols. The changes in de novo synthesis of proteins in cadmium-treated cells were demonstrated by metabolic labeling with [^{35}S]methionine. The data indicated that treatment of cells with different concentrations of cadmium led to the induction of a wide spectrum of stress proteins comprising HSP110, 90, 70s (HSP70 and HSC70), 32, and glucose-regulated protein (GRP) 78 (Fig. 2). However, the level of induction of each stress protein differed considerably, and it was both concentration and time dependent. When the cells were labeled immediately after a 2-h treatment, enhanced synthesis of all the mentioned stress proteins was detected in cells treated with 40 and 60 µM $CdCl_2$, whereas the synthesis of GRP78 was not enhanced in cells treated with 80 and 100 µM $CdCl_2$ (Fig. 2A). After 6–12 h of recovery, enhanced synthesis of the stress proteins, including HSP70 and HSC70, ceased completely in cells treated with 40 µM $CdCl_2$, and the maximal induction of the HSP70s was detected in cells treated with 60, 80, and 100 µM $CdCl_2$ as the recovery period extended (Fig. 2B–D). When the recovery period was prolonged to 16 h, only HSP110 and GRP78 were induced, and enhanced synthesis of these two stress proteins was only detected in cell treated with 100 µM $CdCl_2$ (Fig. 2E). With regard to stress protein induction, the cells appeared to recover completely 24 h after treatment (Fig. 2F). Taken together, the results indicated that enhanced synthesis of stress proteins can be elicited immediately but not after recovery, when the cells were treated with relative low concentrations. However, when the cells were treated high concentrations of cadmium, induction of stress proteins can be detected with or without a recovery period. Furthermore, induction of stress proteins was much more robust in cells treated with higher concentrations than in cells treated with lower concentrations of cadmium.

HSP70 and HSC70 Were Induced in Cadmium-Treated 9L RBT Cells With Different Kinetics

Quantitative analysis of the autoradiograms (Fig. 2) demonstrated the differences in the induction kinetics of HSP70 and HSC70 resulting from the cadmium treatments. The induction kinetics of HSP70 and HSC70 are similar in cells treated with 40 µM $CdCl_2$. Both were induced significantly immediately after the

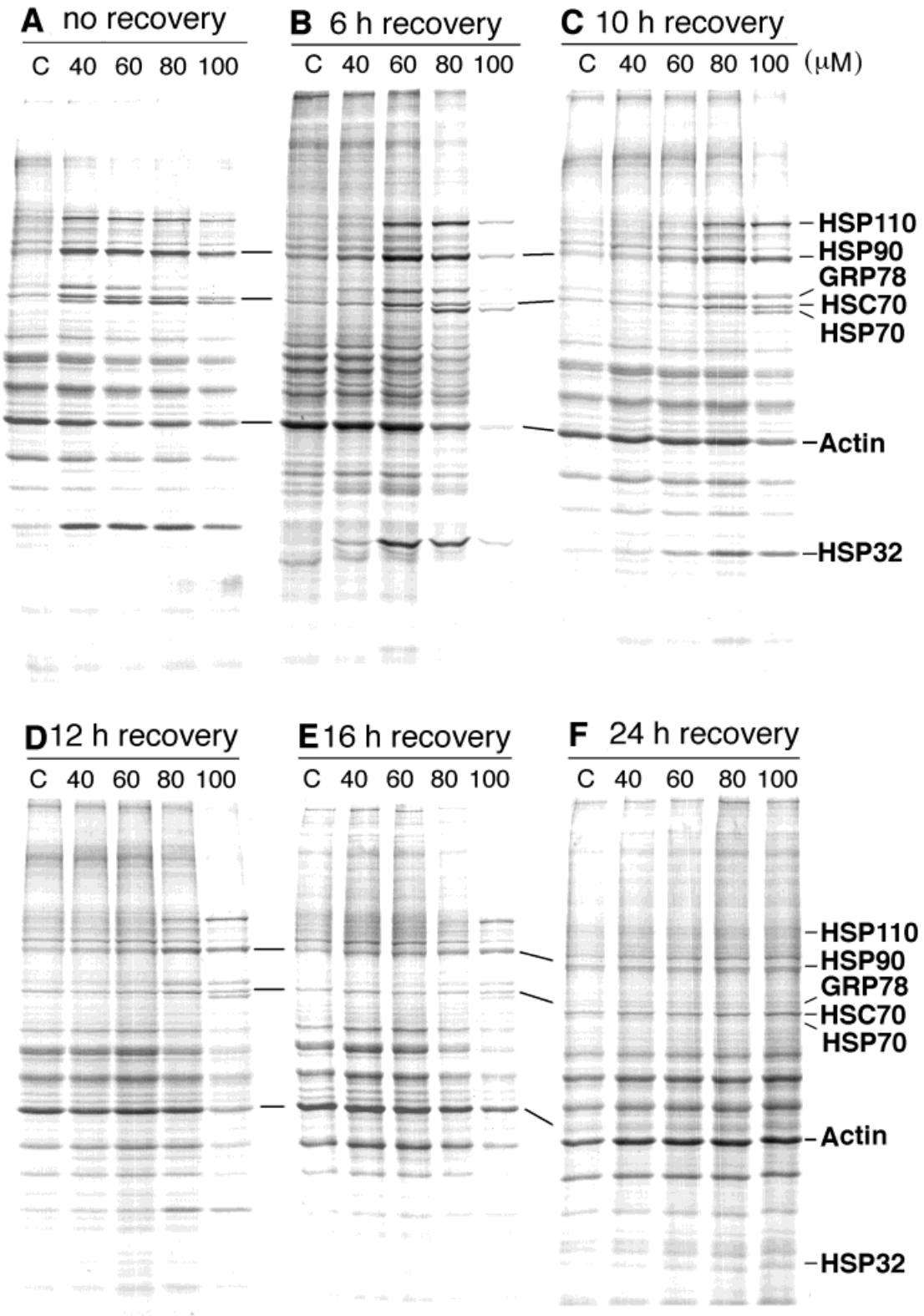


Fig. 2. Induction of stress proteins in cadmium-treated 9L RBT cells. Cells were treated with different concentrations of $CdCl_2$ for 2 h and allowed to recover under normal growing conditions for up to 24 h, as indicated. The cells were then metabolically labeled with [^{35}S]methionine for 1 h before being lysed. The cell lysates were resolved by SDS-PAGE followed by autoradiography. The results indicate that treatment with cadmium results in the induction of HSP110, 90, 70s (HSP70 and HSC70), 32, and GRP78 in 9L cells and that the kinetic of induction is different for each of the stress proteins.

treatment (no recovery), but their rate of synthesis subsided as the treated cells were allowed to recover under normal growing conditions. After 6 h of recovery, the synthesis of HSP70 could no longer be detected and that of HSC70 returned to the basal level of expression (Fig. 3A). In cells that were treated with 60 μM $CdCl_2$, the induction of both proteins was generally greater than that under 40 μM $CdCl_2$ treatment. Moreover, the relative synthesis rates of these two proteins differed from each other significantly, particularly in cells that recovered for less than 8 h (Fig. 3B). When the concentration of $CdCl_2$ was increased to 80 μM or 100 μM, HSP70 became the major stress protein synthesized immediately after and 6–8 h after treatment (Fig. 3C,D). In contrast, the induction kinetic of HSC70 under these conditions differed only slightly from that of the 40 μM $CdCl_2$ treatment. These results indicated that the expression kinetics of HSP70 and HSC70 were similar in cells treated with low concentration of $CdCl_2$ but different in those treated with high concentration of $CdCl_2$. Furthermore, maximum induction of HSP70 was achieved immediately after the treatment in cells treated with low concentrations of cadmium but needed at least 6 h of recovery when the cells were treated with high concentrations of cadmium.

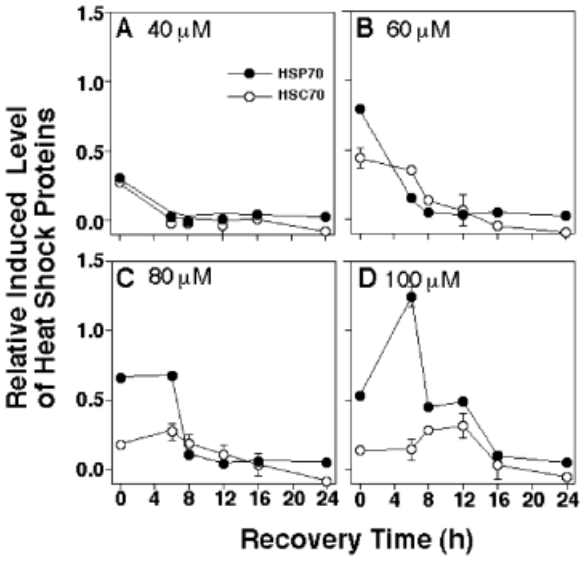


Fig. 3. Kinetics of HSP70 and HSC70 expression in cadmium-treated 9L cells. Bands of HSP70 and HSC70 in autoradiograms, as shown in Figure 2, were quantified by densitometric scanning. The relative synthesis rate of each HSP is presented as the sum of pixel values of each band divided by that of actin in the same lane (internal control) followed by subtraction of the relative units obtained in the control cells. The data are the mean ± SD from three independent experiments. The results demonstrate that the induction of HSP70 and HSC70 by cadmium is both concentration and time dependent.

Hsp70 mRNA and *hsc70* mRNA Accumulate Differently in Cadmium-Treated 9L Cells

To evaluate whether the enhanced expression of HSP70 and HSC70 were transcriptionally regulated, we measured the steady-state accumulation of *hsp70* and *hsc70* mRNAs in cadmium-treated cells by the Northern blotting technique. Cells were treated with 40, 60, and 80 μM of $CdCl_2$ for 2 h and allowed to recover for 6 h before RNA extraction. We found that *hsp70* mRNA was not transcribed in cells under normal condition or in those treated with 40 μM $CdCl_2$, whereas it was highly induced with treatment with higher than 60–80 μM $CdCl_2$ (Fig. 4). By contrast, *hsc70* mRNA was constitutively synthesized, and its synthesis was enhanced slightly in cells treated with 40 μM $CdCl_2$. The enhanced transcription of *hsc70* mRNA reached its maximum in cells treated with 60 μM but subsided in cells treated with 80 μM $CdCl_2$. It should be noted that the changes in *hsp70* and *hsc70* mRNAs synthesis coincided with those of HSP70 and HSC70 protein synthesis determined previously.

Interactions of HSEs Derived From *hsp70* and *hsc70* Promoters With Nuclear Extracts From Cadmium-Treated 9L RBT Cells

The ^{32}P-labeled synthetic HSEs derived from the promoters of *hsp70* and *hsc70*, respectively designated as HSE-*hsp70* and HSE-*hsc70*, were used in the gel shift analysis. Up to four DNA–protein complexes were formed in each of the EMSA studies (Fig. 5). Constitutive formation of complexes A, B, D, and E was detected when HSE-*hsp70* was reacted with nuclear factors from the untreated cells (Fig. 5, lane 2). Except for a decrease in complex D, formation of other complexes remained unchanged when the cells were treated with 40 μM $CdCl_2$ for 2 h and not allowed to recover (Fig. 5, compare lane 3 with lane 2). After 6 h of recovery, initial treatment with 40 μM $CdCl_2$ resulted in a significant increase in complexes A and B and a slight decrease in complex D (Fig. 5, lane 4). When the cells were treated with 80 μM $CdCl_2$ for 2 h and not allowed to recover, formation of complexes A and B was greater but that of complexes D and E was lesser compared with the untreated cells

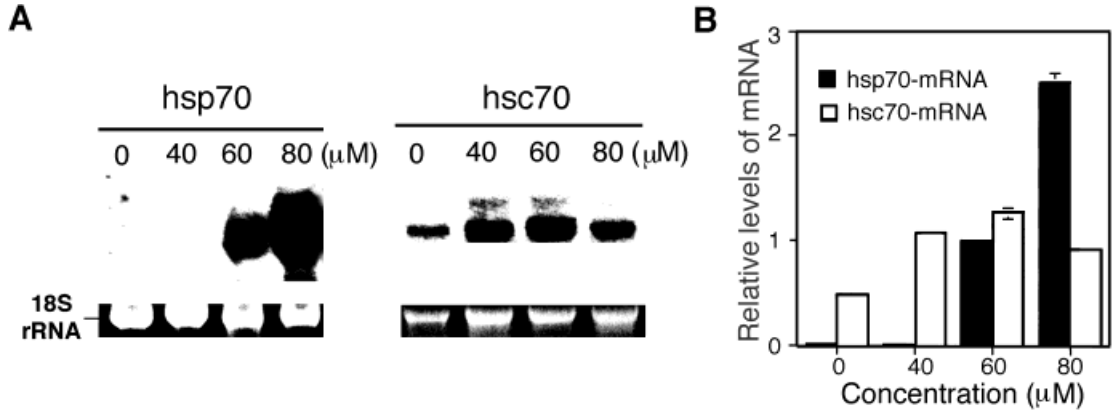


Fig. 4. Concentration-dependent accumulation of mRNA of *hsp70* and *hsc70* in cadmium-treated 9L cells. Cells were treated with 0–80 μM of $CdCl_2$ for 2 h and allowed to recover for 6 h. After recovery, the cells were lysed and total RNA was extracted. **A:** The relative amounts of *hsp70* and *hsc70* mRNA were assessed by Northern blotting. **B:** The relative levels of mRNA were presented as the sums of the pixel values after background subtraction. The data are the mean ± SD from three independent experiments.

(Fig. 5, compare lane 5 with lane 2). After 6 h of recovery, complexes A, B, and D diminished completely, whereas complexes B′ and C′ became detectable (Fig. 5, lane 6). In parallel experiments using HSE-*hsc70* as the EMSA probes, alterations in formation of complexes A, B, and B′ (but not C and C′) were almost identical to those using HSE-*hsp70* as probes (Fig. 5, lanes 8–12). By contrast, formation of complexes D′ and E′ was distinguishable from that of D and E, especially in cells treated with 80 μM $CdCl_2$ for 2 h and not allowed to recover (Fig. 5, lane 11). The specificity of probe-binding activities was investigated further by competitive assays. When using identical nonradioactive oligonucleotide sequences as competitors, all of the complex formations were abolished (Fig. 6A). Furthermore, when nonradioactive HSE-*hsc70* was used to compete the preformed HSE-*hsp70*–protein complexes, only complexes A and B could be chased by the addition of excess unlabeled competitor, whereas the formation of complexes D and E remained unaltered (Fig. 6B, left). Reciprocally, complexes A and B but not complexes D′ and E′ in preformed HSE-*hsc70*–protein reactions could be chased by the addition of excess unlabeled HSE-*hsp70* (Fig. 6B, right). Taken together, these results demonstrated that the nuclear extracts from the control and cadmium-treated cells contain overlapping but distinctly different factors that are able to form DNA–protein complexes with HSEs derived from *hsp70* and *hsc70*, respectively.

Interactions of Basal Transcription Elements Derived from *hsp70* and *hsc70* Promoters With Nuclear Extracts From Cadmium-Treated 9L RBT Cells

The roles of several well-known basal transcription elements including TATA, GC, and CCAAT boxes in the transcriptional regulation of the *hsp70* and *hsc70* in the cadmium-treated cells were also analyzed by EMSA. Synthetic oligonucleotides derived from *hsp70* and *hsc70* promoters (Fig. 1) were used. The probes were designated as TATA-*hsp70*, GC-*hsp70*, CCAAT-*hsp70*, TATA-*hsc70*, GC-*hsc70*, and CCAAT-*hsc70*, respectively. Cells treated with 60 μM $CdCl_2$ for 2 h were used to prepare the nuclear extracts. Five DNA–protein complexes were formed, and the same complexes were detected when using TATA-*hsp70* and TATA-*hsc70* as EMSA probes. However, the intensity of TATA-*hsp70* was heavier than that of TATA-*hsc70*, and there was no difference in band mobility except for the second band next to the bottom (Fig. 7A, arrow). The bindings of the nuclear factors to GC-*hsp70* and GC-*hsc70* were also different, as indicated by the differences in the intensity (the upper two bands) and the positions (marked by arrows) of the DNA–protein bands (Fig. 7B). Differences were also found between the untreated and treated cells when GC-*hsp70* was used as the probe (Fig. 7B, compare lane 2 with lane 3), but there was no difference when GC-*hsc70* was used (Fig. 7B, lanes 5 and 6). The differences in probe binding

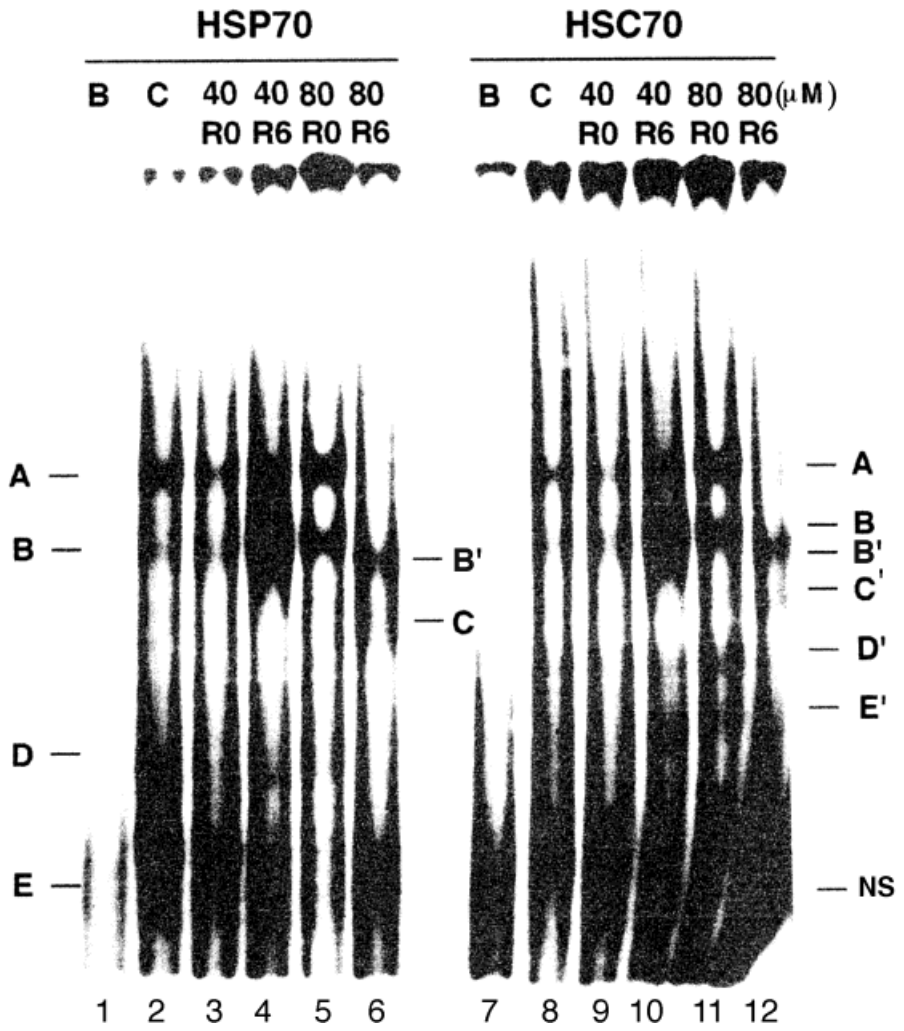


Fig. 5. Interaction of nuclear factors extracted from cadmium-treated 9L cells with the HSEs from the promoter region of *hsp70* and *hsc70*. Nuclear extracts were prepared from cells that were treated with 40 or 80 μM $CdCl_2$ for 2 h followed by 0 (R0) or 6 (R6) h of recovery. Synthetic oligonucleotides corresponding to the HSEs of the promoter fragments of *hsp70* and *hsc70*, as indicated in Figure 1, were annealed, endlabeled, and mixed with the nuclear extracts. DNA–protein complexes were analyzed by EMSA. The positions of the specific DNA–protein complexes A, B, B′, C, C′, D, and D′ are indicated. Similar results were observed in three independent experiments.

also applied when CCAAT boxes were used. The bindings of the nuclear factors to CCAAT-*hsp70* and CCAAT-*hsc70* were different as demonstrated by the differences in the intensity and positions (marked by arrows) of the DNA-protein bands (Fig. 7C). However, when the treated and untreated cells were compared, the differences in probe binding were found in CCAAT-*hsc70* (Fig. 7C, lanes 5 and 6) but not in CCAAT-*hsp70* (Fig. 7C, lanes 2 and 3) samples, opposite to the findings of GC boxes. Taken together, these data suggested that all of the basal elements, i.e., TATA, GC, and CCAAT boxes, are involved in the differential transactivation of the heat shock genes under our experimental conditions.

Interactions of Extended *hsp70* and *hsc70* Promoter Fragments With Nuclear Extracts From Cadmium-Treated 9L RBT Cells

We also employed the extended promoter fragments of the heat shock genes as EMSA probes to study the binding pattern in the transactivation of *hsp70* and *hsc70* in the cadmium-treated cells.. The PCR products generated by the primers (Fig. 1) were respectively designated as promoter-*hsp70* and promoter-*hsc70*. The radioactive EMSA probes were allowed to react with

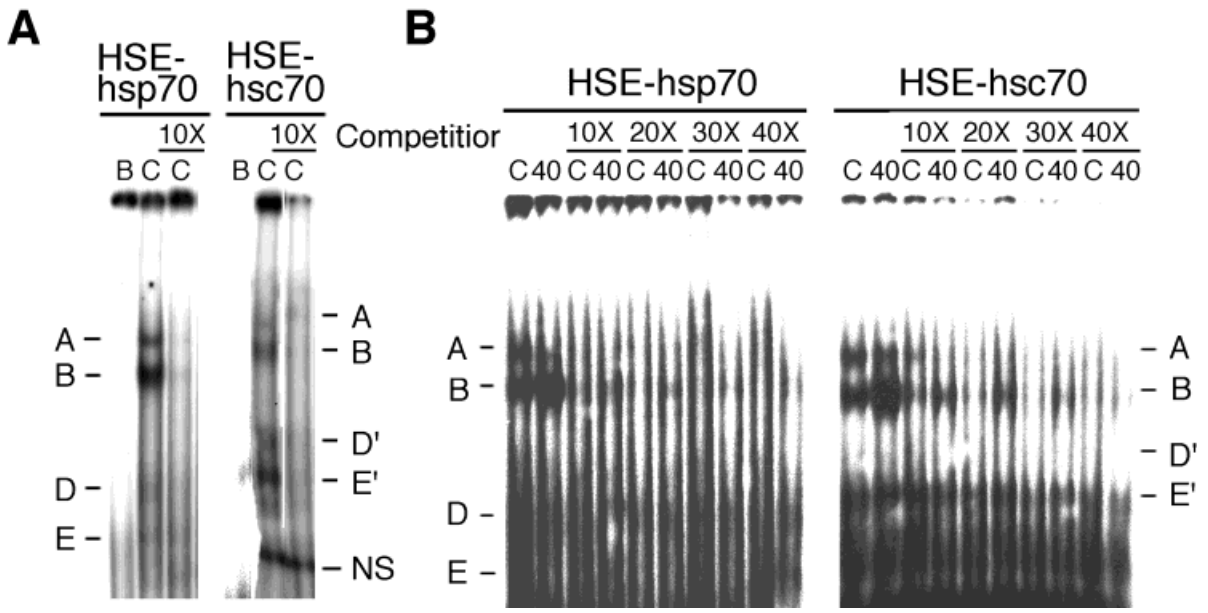


Fig. 6. Competitive assays of interaction of nuclear factors extracted from cadmium-treated 9L cells with the HSE-*hsp70* and HSE-*hsc70*. Nuclear extracts were prepared from cells that were treated with 0–80 μM $CdCl_2$ for 2 h with or without 6 h of recovery. Synthetic oligonucleotides corresponding to the HSEs of the promoter region of *hsp70* and *hsc70* were annealed, endlabeled, and mixed with the nuclear extracts. The DNA–protein complexes were analyzed by EMSA. **A:** The binding of nuclear factors to the probes was assessed by adding excessive amounts (10-fold molar ratio) of nonradioactive probes in the reactions. **B:** The binding activities were analyzed further by adding nonradioactive HSE-*hsc70* probe to the preformed protein–HSE-*hsp70* complexes and vice versa. **Lane B** contained only labeled DNA (no nuclear extract). **Lane C** contained nuclear extract from untreated cells. The positions of the DNA–protein complexes are indicated. NS, nonspecific signal. Similar results were observed in three independent experiments.

the nuclear extracts with or without sequential addition of 100× excess of specified unlabeled basal elements in the following order—TATA, GC, and then CCAAT (Fig. 8)— presumably leading to specific removal of the corresponding basal transcription factors, namely TFIID, Sp1, and CCAAT binding factor (CBF). Three DNA–protein complexes, designated as I, II, and III, formed when nuclear extracts from untreated cells were allowed to reacted with promoter-*hsp70* (Fig. 8A, left, lane 1). During the sequential removal of the transcription factors, formation of complexes II and III diminished consequentially (Fig. 8A, left). It was also noted that complex II could be completely abolished when excess unlabeled HSE-*hsp70* was added (Fig. 8A, left, lane 4). Moreover, an enhanced formation of complex I appeared when all of the basal transcription factors were removed, and this complex persisted even when an excess amount of unlabeled HSE-*hsp70* was included (Fig. 8A, left, lanes 4 and 5). When promoter-*hsc70* was used in the same reaction, removal of Sp1 by adding an excess amount of GC-*hsc70* resulted in increased formation of all the DNA–protein complexes (Fig. 8A, right, lane 3), which, except complex I, were completely diminished by adding an excess amount of HSE-*hsc70* (Fig. 8A, right, lane 5). Alterations in binding activities were significantly different in the nuclear extracts from cadmium-treated cells. A newly formed DNA–protein complex, designated as II′, and complex I were detected, and the binding activities toward promoter-*hsp70* were higher than promoter-*hsc70* when the promoter fragments were allowed to react with nuclear extracts prepared from cells treated with 80 μM $CdCl_2$ without recovery (Fig. 8B). Moreover, sequential removal of the basal transcription factors did not seem to affect the formation of these complexes (Fig. 8B). When the treated cells were allowed to recover for 6 h, only complex III was detected in all samples, and the formation of this complex was also not affected by the removal the basal transcription factors (Fig. 8C). The data implied that the basal transcription elements in addition to HSF-HSE binding may play important and distinguishable role(s) in the transactivation of the heat shock genes in cadmium-treated 9L RBT cells.

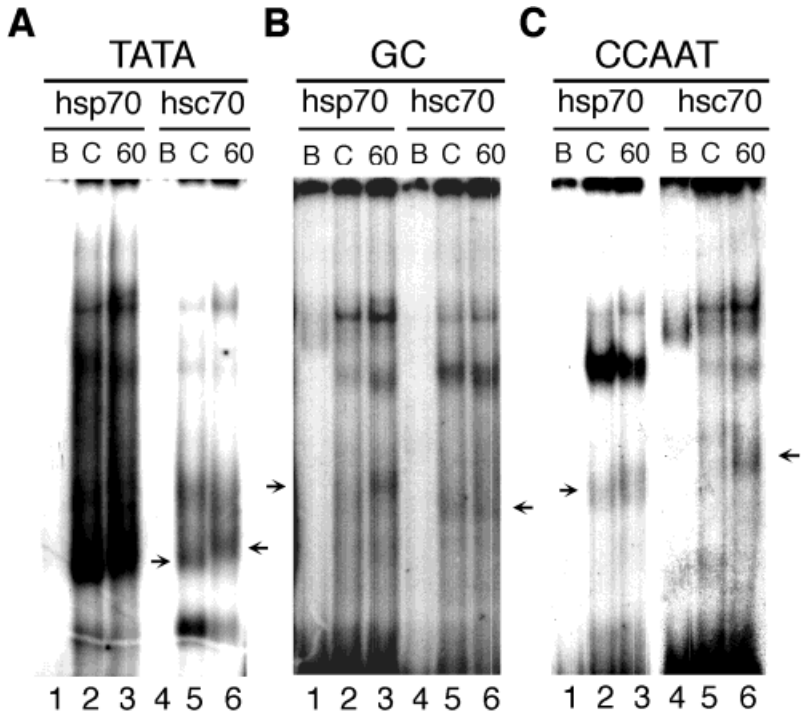


Fig. 7. Interaction of nuclear factors extracted from cadmium-treated 9L cells with the regulatory elements TATA, GC, and CCAAT boxes from the promoter region of *hsp70* and *hsc70*. Nuclear extracts were prepared from cells that were treated with 60 μM $CdCl_2$ for 2 h without recovery (**A**) or with 6 h of recovery (**B**). Synthetic oligonucleotides corresponding to the regulatory elements including TATA, GC, and CCAAT boxes of the promoter region of *hsp70* and *hsc70* were annealed, endlabeled, and mixed with the nuclear extracts. The DNA–protein complexes were analyzed by EMSA. **Lane B** contained only labeled DNA (no nuclear extract). **Lane C** contained nuclear extract from untreated cells. Similar results were observed in three independent experiments.

DISCUSSION

Induction of HSPs and enhanced expression of GRPs involve different signaling systems that are usually mutually exclusive [Watowich and Morimoto, 1988]. Interestingly, treatment of 9L RBT cells with cadmium leads to the induction of a wide spectrum of stress proteins comprising both HSPs and GRPs. This observation indicated that the metal ion is capable of activating different signaling pathways leading to the activation of a battery of transcription factors that are involved in the regulation of the stress genes. With regard to the HSP70s, we have shown that treatment of 9L RBT cells with $>$40 μM $CdCl_2$ results in the differential induction of the HSP70s, whose expressions are governed by the distinguished promoter activities involving the HSE and a number of basal transcription elements. In cells exposed to low dosage of cadmium, induced synthesis of the protective factors such as metallothionein and glutathione confers the acquired tolerance to the metal ion [Nordberg, 1978; Eaton et al., 1980]. However, treatment with cadmium at cytotoxic level is required for the induction of stress proteins such as HSP70 and HSC70 [Ovelgonne et al., 1995]. In the present studies, treatment of cells with $<$40 μM $CdCl_2$ for 2 h did not lead to induction of any of the stress proteins (data not shown), but induction of a wide spectrum of stress proteins was detected after the cells were exposed to $>$40 μM $CdCl_2$ for 2 h, indicating that treatment of 9L RBT cells at 40 μM $CdCl_2$ for 2 h may have overwhelmed the protective functions provided by intracellular metallothionein and glutathione; thus, this treatment condition represents the threshold for stress protein induction. Although both HSP70 and HSC70 can be induced by cadmium in 9L RBT cells, several lines of evidence suggest that the expression of these two HSPs are governed by distinguished regulatory mechanisms. First, HSC70 but not HSP70 is constitutively expressed in unstimulated cells. Second, enhanced synthesis of HSC70 can be achieved under milder conditions, but induc-

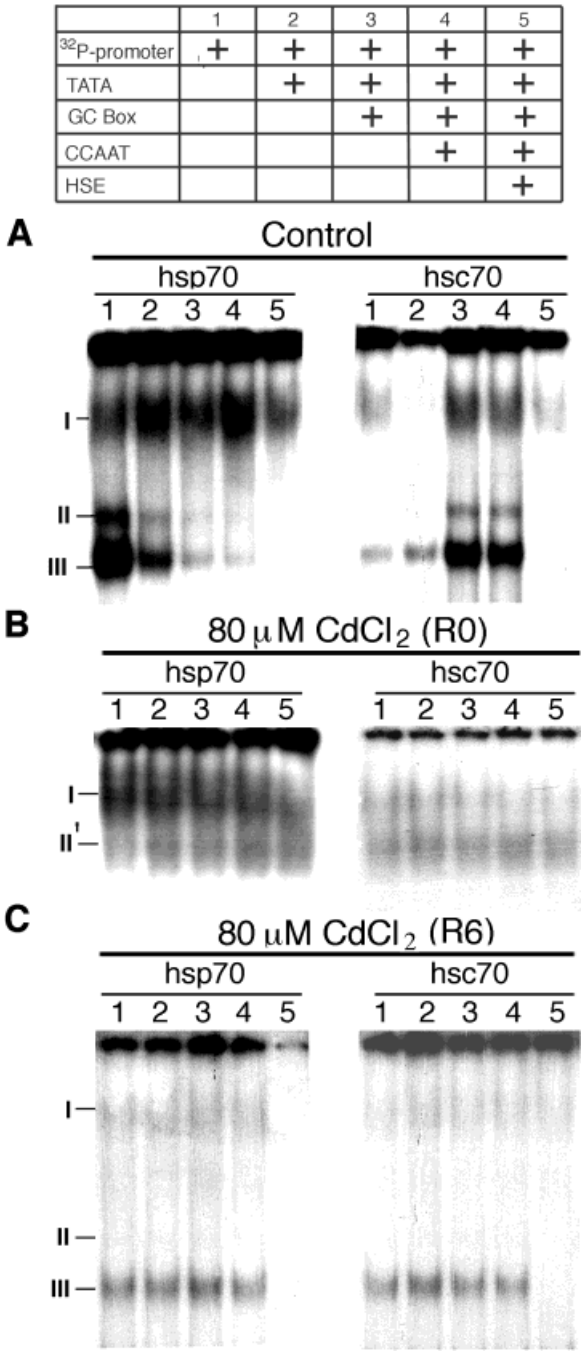


Fig. 8. Interaction of nuclear factors extracted from cadmium-treated 9L cells with the extended promoter regions of *hsp70* and *hsc70*. Nuclear extracts were prepared from untreated cells (**A**) or cells that were treated with 80 μM of $CdCl_2$ as indicated for 2 h without recovery (**B**) or with 6 h of recovery (**C**). The promoter region of *hsp70* and *hsc70* were generated by PCR, endlabeled, and mixed with the nuclear extracts. Sequential addition of the excess unlabeled oligonucleotides corresponding to respective transcription elements is indicated at the top of the figure. DNA–protein complexes were analyzed by EMSA. The positions of the specific DNA–protein complexes I, II, II′, and III are indicated. Similar results were observed in three independent experiments.

tion of HSP70 requires much more severe stress treatment. Third, the induction of HSP70 is generally massive, sudden, and long lasting, whereas enhanced synthesis of HSC70 is always milder, gradual, and short lasting. Biochemically, we previously demonstrated that, in heat shocked cells, HSC70 is associated with intermediate filaments and that HSP70 is associated with the tubulins, the building blocks of microtubules [Lee et al., 1992; Cheng and Lai, 1994]. By using immunofluorescence microscopy, we have shown that accumulation of HSC70 is more transient, and the protein translocated to and subsequently exited from the nucleus more rapidly than did HSP70 [Wang et al., 1998]. These observations and the differential expression kinetics suggest that HSP70 and HSC70 may be not physiologically identical, despite the fact that both proteins may function as molecular chaperones in stressed cells.

Transactivation of HSE-containing heat shock genes primarily involves HSE–HSF complex formation [Bienz and Pelham, 1986; Peristic et al., 1989]. In general, HSF exists in an inactive state and becomes activated to cooperate with HSE for transcription of heat shock genes with exposure of cells to physiological stresses [Kingston et al., 1987; Abravaya et al., 1991]. However, it has also been shown that binding of HSF to HSE does not necessarily result in the transactivation of the heat shock genes [Liu et al., 1993; Mathur et al., 1994]. By using HSE-*hsp70* and HSE-*hsc70* as EMSA probes, we have demonstrated that HSE–protein complexes can be formed in rat cells with or without cadmium treatment, and this observation supports the notion that HSE–HSF formation is a required but not a sufficient process for heat shock gene transactivation. We have also found that continuous binding of HSF to HSEs is not required for the sustained expression of HSP70s. For instance, in cells that were treated with 80 μM $CdCl_2$ and allowed to recover for 6 h while both HSP70 and HSC70 were still being vigorously synthesized, HSF was found to be dissociated from the HSEs. A number of transcription factors, including mammalian HSF, use phosphorylation as a major regulatory process for nuclear localization, DNA binding, transcriptional activation, association with other factors, and degradation [Hunter and Karin, 1992; Jackson, 1992; Karin, 1994]. Constitutive phosphorylation of HSF1 at serine residues distal to the transcriptional activation domain has

been shown to function to repress transactivation while that inducible phosphorylation correlates with transcriptional activation [Cotto et al., 1996; Kline and Morimoto, 1997]. Whether or not phosphorylation is involved the regulation of HSF in cadmium-treated cells is not known and warrants further investigation.

In addition to HSE–HSF interaction, heat shock genes are also regulated by a complex interplay involving a number of transcription elements and factors. It has been shown that maximal stress-induced transcription from the human *hsp70* promoter required the interactions of CBF and Sp1 with HSF [Morgan et al., 1987; Williams and Morimoto, 1990]. In addition, thermal induction of human HSP70 expression is modulated by a constitutive HSE binding factor (CHBF) [Yang et al., 1996]. In the present studies, differential binding activity of transcription factors to *hsp70* and *hsc70* promoters are identified not only by HSEs but also by basal transcription elements TATA, GC, and CCAAT box. These results indicated that the binding activities of a set of transcription elements rather than HSE alone is responsible for the differences in basal expression and for inducibility of the *hsp70* and *hsc70* genes in 9L RBT cells. In other words, the distinct regulatory mechanisms between these two closely related genes must be achieved by the participations of HSF/CHBF, Sp1, CBF, and perhaps other factors that bind to HSE, GC, and CCAAT. The exact interplay of these transcription factors in the differential transactivation of rat *hsp70* and *hsc70* needs to be elucidated further.

In summary, we have demonstrated that treatment of 9L RBT cells will lead to the transactivation of a number of stress genes including *hsps* and *grps*. We have analyzed the promoter activities of *hsp70* and *hsc70* under different treatment conditions and have identified the activation of a number of transcription factors that are involved in the differential induction of these two HSPs. Further studies on the functionalities and cooperativities of the involved transcription elements and corresponding transcription factor in this experimental system may provide detailed regulatory mechanisms governing the expression of the stress genes.

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