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Differential expression of two stress-inducible *hsp*70 genes by various stressors

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Abstract

Inducible HSP70s encoded by two almost identical *hsp*70 genes, known as *hsp*70.1 and *hsp*70.3 are located in a tandem array on mouse chromosome 17. Duplication of this gene has been found in various higher organisms. However, the role of two almost identical *hsp*70 genes is still unclear. To elucidate the ambiguity of expression pattern between *hsp*70.1 and *hsp*70.3, we investigated the induction of each *hsp*70 isoforms by several HSP70-inducible stressors in wild type (*hsp*70.1+/+ and *hsp*70.3+/+) and the *hsp*70.1-/- (*hsp*70.1-/- and *hsp*70.3+/+) murine embryo fibroblast (MEF) cells, and the M-1 mouse cortical collecting duct cell line. Each induced *hsp*70 isoforms by heat shock were very similar at transcriptional and translational levels in wild type and *hsp*70.1-/- MEF cells. The mRNA stabilities of both *hsp*70.1 and *hsp*70.3, even in two kinds of *hsp*70.3 transcripts, were also very similar. L-azetidine-2-carboxylic acid, sodium arsenite, $CdCl_2$ and $ZnCl_2$ caused induction of both isoforms of the *hsp*70 genes, even though their expression levels were variable. NaCl caused induction of just *hsp*70.1 gene expression. H_2O_2 failed to induce the expression of either *hsp*70 genes in MEF cells, caused induction of *hsp*70.1 gene expression in the M-1 cell line. Hsp70 accumulation by H_2O_2 and NaCl treatment was mainly due to *hsp*70.1 expression. Our studies demonstrated that two *hsp*70 genes respond differentially to types of stress.

Keywords: *hsp*70.1, *hsp*70.3, induction, MEF cells, stressor

Introduction

The 70 kDa heat shock proteins (HSPs) are one of the most conserved proteins from bacteria to human (Schuh *et al*., 1985; Tanaka *et al*., 1988). Whereas HSC70 is constitutively expressed, HSP70 can be induced by various stresses such as hyperthermia, oxidative stress, heavy metals and amino acid analogues (Morimoto *et al*., 1992; Abe *et al*., 1998; Fukamachi *et al*., 1998; Wagner *et al*., 1999). HSP70 has an important function as a molecular chaperone for the folding, transport and assembly of newly synthesized polypeptides (Hartl, 1996). Moreover, it has been reported to protect cells from a number of apoptotic stimuli (Jäättelä *et al*., 1992; Mosser and Martin, 1992; Mailhos *et al*., 1993; Simon *et al*., 1995; Samali and Cotter, 1996; Ahn *et al*., 1999; Beere *et al*., 2000; Li *et al*., 2000; Saleh *et al*., 2000). HSP70 is encoded by two major stress-inducible *hsp*70 genes, *hsp*70.1 and *hsp*70.3 which are located in a tandem array on the major histocompatibility complex (MHC) region of the mouse chromosome 17 (Hunt *et al*., 1993; Snoek *et al*., 1993). Linkage between MHC and inducible *hsp*70 genes has been shown in many species including rat and human (Walter *et al*., 1994). Because the two mouse HSP70 isoforms are nearly identical to each other, differing only at two amino acids residues, they cannot be distinguished even by two dimensional SDS-PAGE (Dix *et al*., 1998), and their expression patterns and distinguishable functions are still unknown.

In this study, to resolve ambiguity of expression pattern between *hsp*70.1 and *hsp*70.3, we investigated the induction profile of *hsp*70.1 and *hsp*70.3 at the protein level, and monitored their mRNAs with *hsp*70.1 and *hsp*70.3 specific probes, in *hsp*70.1+/+ and *hsp*70.1-/- MEF cells and in the M-1 mouse cortical collecting duct cell line by various stressors including heat shock, heavy metals, amino acids analogue, hyperosmolarity and oxidative stress. From our results, we conclude that *hsp*70.1 has a more pivotal role in stress response than *hsp*70.3 and two *hsp*70 genes respond differentially to different stress types.

Materials and Methods

Chemicals and reagents

$ZnCl_2$, $CdCl_2$, NaCl, L-azetidine-2-carboxylic acid, and sodium arsenite were obtained from Sigma (St. Louis, MO, USA). The molecular weight standards for SDS-PAGE were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Enhanced chemiluminescence (ECL) reagent for western blotting detection was

purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Anti-HSP70 antibody which was specific for HSP70 and non cross-reactive with HSC 70 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies, anti-mouse IgG conjugated to horseradish peroxidase (HRP) were obtained from Pierce (Rockford, IL, USA).

Cell culture and treatment

MEF cells were prepared from C57BL/6 normal mice embryos and *hsp*70.1 knockout mice embryos in the C57BL/6 genetic background at embryonic day 13.5 postcoitum. Cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 50 μg/ml penicillin and streptomycin in a 5% CO_2 incubator. M-1 cells were grown in the same condition except a supplement with 5% heat-inactivated fetal bovine serum.

For heat shock conditions, culture plates containing cells were tightly wrapped with parafilm and immersed in a water bath at the indicated temperature for 30 min, then kept in CO_2 incubator at 37°C for indicated times. Other stress conditions were induced by which cells were kept in media containing zinc chloride, cadmium chloride, sodium chloride, L-azetidine-2-carboxylic acid, sodium arsenite at indicated concentrations for 6 to 24 h.

Construction of specific probes for *hsp*70.1 and *hsp*70.3 genes

To construct specific probes of *hsp*70.1 and *hsp*70.3 each for Northern blot analysis, the following two specific primer sets were used in the polymerase chain reaction (PCR): the sense and antisense primers for *hsp*70.1 are 5'-TGCACTTGATAGCTGCTTGG and 5'-GCAGTGTAGACATGTATGCA, the sense and antisense primers for *hsp*70.3 are 5'-CTGGCTAGGAGACAGATATG and 5'-GGGCAGTGCTGAATTGAAGA, respectively. The resulting PCR fragments were inserted into the pTBlue(R) vector (Novagen, WI, USA). Both constructs were verified with sequencing reaction and digested with *Hind*III-*Eco*RI for preparing specific probes.

RNA isolation and Northern blot analysis

Total RNA was isolated with RNAgents® Total RNA Isolation System (Promega, WI, USA). For Northern blot analysis, 10 μg of total RNA was fractionated on a 1% formaldehyde agarose gel, blotted onto Hybond-N+ nylon membrane (Amersham, NJ, USA). The ^{32}P-labeled probes by random priming kit (Amersham, NJ, USA) were hybridized to the blots at 62°C in a Modified Church Hybridization Buffer (0.25 M Na_2HPO_4, 1 mM EDTA, 1% casein, 7% SDS, adjust pH to 7.4 with H_3PO4). The blots were washed under high stringency and autoradiographed on the X-ray films (AGFA, Germany).

Preparation of whole cell extract and Western blot analysis

After washing with phosphate-buffered saline, the cells were scraped and collected in extraction buffer (1% Triton X-100, 1% sodium deoxycholate, 25 mM Tris-HCl, pH 7.4, 0.1% SDS, 137 mM NaCl, 1 mM EDTA). The collected cells were incubated on ice for 30 min. The lysate was centrifuged and the protein amount in cleared lysates was quantitated with BCA Protein Assay Reagents (Pierce, IL, USA). The equal amounts of total proteins were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. HSP70 band was detected by sequential treatment with an anti-HSP70 antibody, an HRP-conjugated secondary antibody, and an enhanced chemiluminescence substrate kit.

Results

Both *hsp*70.1 and *hsp*70.3 genes were induced with similar expression patterns by heat shock in MEF cells

In order to investigate the *in vivo* function of the *hsp*70.1 gene, *hsp*70.1 knockout mice were generated in our laboratory. The successful targeted integration of the knockout construct was confirmed by Southern blot analysis (Lee *et al.*, 2001). To ensure specific disruption

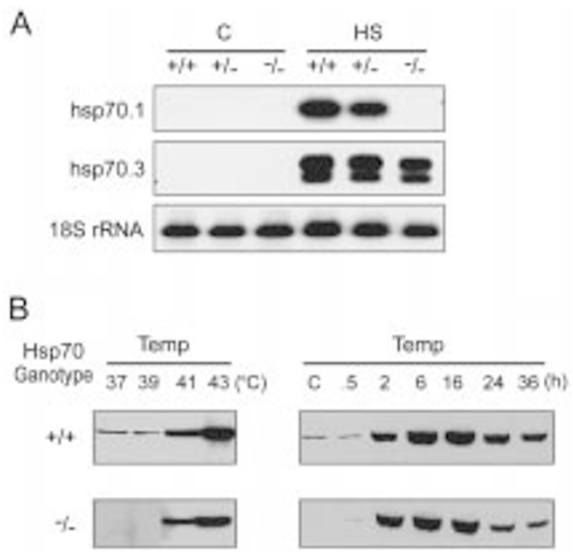

Figure 1. Induction pattern of HSP70 in *hsp*70.1+/+ and *hsp*70.1-/- MEF cells. A. Northern analysis of *hsp*70.1-/- MEF cells. Total RNAs were isolated from *hsp*70.1 wild type (+/+), hetrozygote (+/-), and homozygote (-/-) cells untreated (C) or treated with heat shock (43ºC, 30 min) followed by 2 h recovery (HS). Hybridization was performed with *hsp*70.1 and *hsp*70.3 specific probes obtained by PCR as described in Materials and Methods. 18s rRNA was used as a control for the equal loading of total RNAs. B. Temperature- and time-dependent expression of HSP70 in *hsp*70.1+/+ and -/- MEF cells by immunoblot assay. Time indicates recovery duration (hours) at 37ºC after heat treatment.

of *hsp*70.1 and not of *hsp*70.3, we constructed specific probes for *hsp*70.1 and *hsp*70.3, as described in Materials and Methods. Since the 3'-untranslated region (UTR) of inducible *hsp*70 genes shows low similarity, in contrast to the high sequence similarity within the coding regions of *hsp*70 in both interspecies and intraspecies (Hunt *et al*., 1993), we amplified part of the 3'-UTR of each inducible *hsp*70 gene by PCR. The Northern blot analysis with the specific probes for *hsp*70.1 and *hsp*70.3 in *hsp*70.1-/- MEF cells showed that the *hsp*70.1 gene was specifically interrupted (Figure. 1A). Northern analysis of *hsp*70.3 shows its two transcripts differing 3'-ends, as reported by Perry *et al*. (1994). Since it has not been clearly reported whether both inducible *hsp*70 genes contribute equally to HSP70 induction by stress or not, we first investigated the induction level of each *hsp*70 gene by heat shock, which is known to be the most general stress for HSP70 accumulation, in *hsp*70.1-/- MEF cells. When cells were exposed to a heat shock condition, both *hsp*70.1 and *hsp*70.3 genes were induced rapidly, dramatically and evenly (Figure 1B). However, the level of inducible HSP70 in the unheated *hsp*70.1-/- MEF cells was undetectable. It means that the basal level of HSP70 is mainly due to *hsp*70.1 expression. The basal level of inducible HSP70 in almost all organs of *hsp*70-/- mice was also remarkably reduced in comparison with wild type mice (our unpublished data). Therefore, we hypothesized that *hsp*70.1 may have an important role in overcoming the mild stresses encountered in normal circumstance, which doesn't warrant the induction of HSP70.

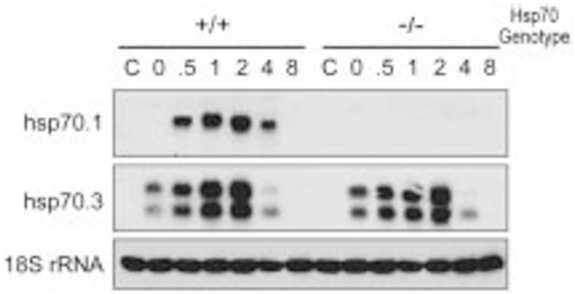

Figure 2. The stability of *hsp*70.1 and *hsp*70.3 transcripts after heat shock treatment in *hsp*70.1+/+ and -/- MEF cells. MEF cells were exposed to heat shock condition (43°C, 30 min) and then recovered at 37°C for indicated time periods. Northern blot analysis was performed with specific probes for each gene. C denotes untreated MEF cells. Numbers indicate recovery duration after heat shock. 18s rRNA was used as a control for the equal loading of total RNA. Two bands shown in the blot hybridized with an *hsp*70.3 specific probe indicate two transcripts of *hsp*70.3 with different polyadenylation sites.

We next examined the mRNA levels of the two *hsp*70 genes by heat shock. The induction of *hsp*70 genes was observed at the transcript level using Northern blot analysis with a specific probe for each transcript. No significant difference was observed in their inducibilities and stabilities, even in the two transcripts of *hsp*70.3 (Figure 2).

Two inducible *hsp*70 genes were differentially induced by stress types in MEF cells

Since there was no detectable difference in response of *hsp*70 genes to heat shock at the mRNA and protein

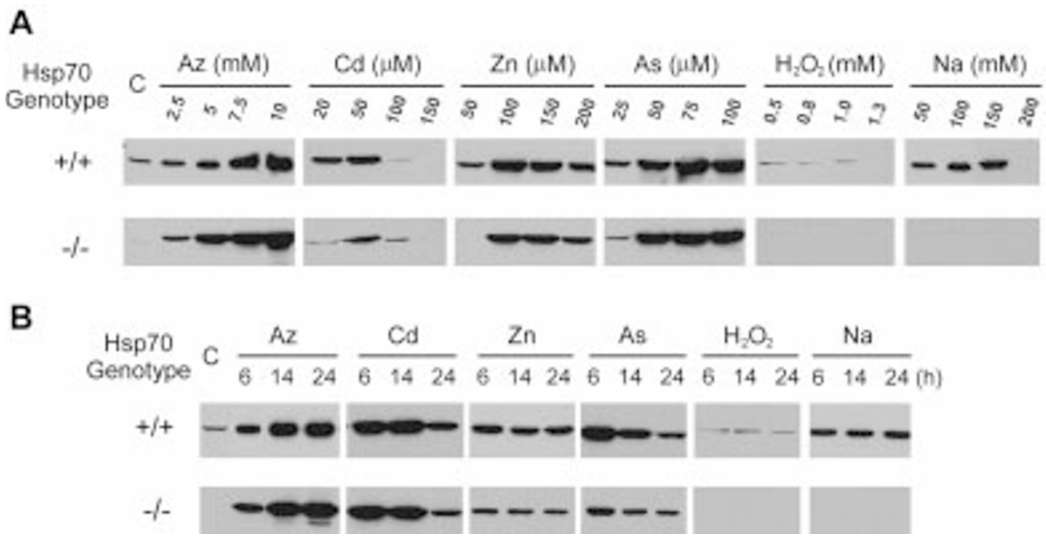

Figure 3. Concentration- and time-dependent expression patterns of HSP70 on amino acids analogue, heavy metals, oxidative stress, hyperosmotic stress. A. Concentration-dependent patterns of HSP70 expression. Whole cell lysates were obtained by incubating *hsp*70.1+/+ and -/- MEF cells with various concentrations of each stressor for 14 h and analyzed by immunoblot assay. Each number indicates final concentration of stressors. C denotes untreated MEF cells. Az, Cd, Zn, As, H_2O_2, and Na refer to MEF cells which were treated with L-azetidine-2-carboxylic acid, cadmium chloride, zinc chloride, sodium arsenite, hydrogen peroxide and sodium chloride, respectively. B. Time-dependent patterns of HSP70 expression. Numbers indicate treatment duration of stressors. The final concentration of each stressor used for HSP70 induction were as follows: Az, 5 mM; Cd, 50 μM; Zn, 100 μM; As, 50 μM; H_2O_2, 0.8 mM; and Na, 100 mM.

level, we wanted to know whether the two very similar genes are subject to differential regulation by stress type. Wild type and *hsp*70-/- MEF cells were treated with various stressors including heavy metals ($CdCl_2$, $ZnCl_2$, sodium arsenite), amino acids analogue (L-azetidine-2-carboxylic acid), oxidative stress (H_2O_2) and hyperosmotic stress (NaCl). $CdCl_2$, $ZnCl_2$, sodium arsenite, and L-azetidine-2-carboxylic acid induced HSP70 with respect to the different concentration and treatment duration of the stressors both in the control and in *hsp*70.1 -/- MEF cells (Figure 3A and 3B). On the contrary, hyperosmolarity specifically induced the *hsp*70.1 gene and oxidative stress induced neither of the *hsp*70 genes. The highest concentration of $CdCl_2$ and NaCl was too high to induce HSP70 accumulation and drove cells into the almost dead state. These results were confirmed by Northern blot analysis as shown in Figure 4. Heat, sodium arsenite, and L-azetidine-2-carboxylic acid induced both mRNAs of *hsp*70, though there were differences in the levels of mRNA inducibility (Figure 4A). The two transcripts of *hsp*70.3 responded almost identically under the tested stress conditions. Since mRNA expression by H_2O_2 was undetectable and the mRNA induced by NaCl was too low after 2 h of treatment, we decided to observe the mRNA level at 6 h and 12 h after treatment with either H_2O_2 or NaCl. Results were consistent with the results of protein induction (Figure 4B). The *hsp*70.1 level was dramatically increased in cells treated for 6 and 12 h with NaCl, but *hsp*70.3 induction was not detected. H_2O_2 did not induce either *hsp*70 gene at the transcript or protein levels. When MEF cells were treated with H_2O_2 for longer than 12 h, the same result was obtained (data not shown).

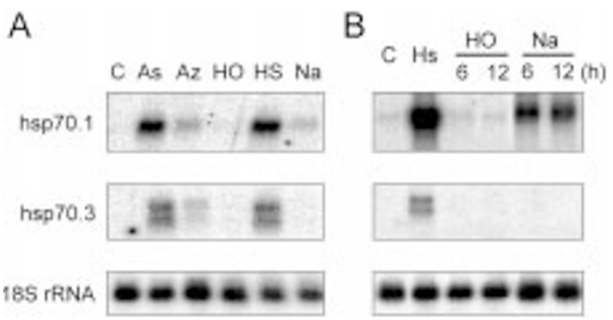

Figure 4. Northern blot analysis using *hsp*70.1- and *hsp*70.3-specific probes of MEF cells treated with various stressors. A. Total RNAs were isolated from the MEF cells untreated (C) or treated with each stressor for 2 h. The concentrations of the stressors were detailed in Figure 3B. B. Total RNAs were isolated from MEF cells treated with 0.8 mM H_2O_2 or 100 mM NaCl for 6 or 12 h. HS denotes MEF cells which were heat shock treated, and then allowed to recover at 37°C for 2 h. 18s rRNA was used as the control to show equal loading of total RNA.

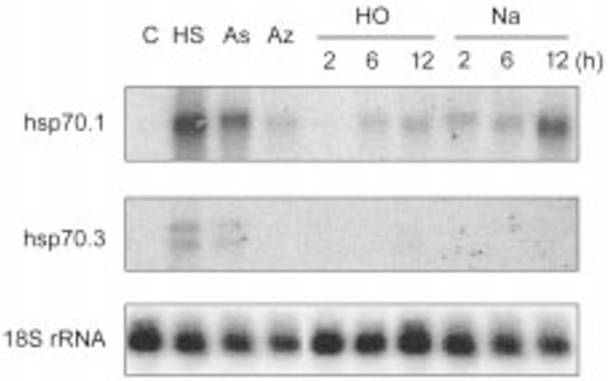

Figure 5. Northern blot analysis using *hsp*70.1- and *hsp*70.3-specific probes of M-1 cells treated with various stressors. Total RNAs were isolated from M-1 cells untreated (C) or treated with each stressor for 2 h, 6 h or 12 h. The type and the concentrations of stressors were shown in the legend of Figure 3.

Only *hsp*70.1 gene responded to stresses of hydrogen peroxide and sodium chloride in the M-1 cell line

To identify whether the inducibilities of *hsp*70 genes show different patterns in different cell types, we performed Northern blot assay using the M-1 mouse cortical collecting duct cell line. The responses to the treatment of heat, sodium arsenite and sodium chloride were similar to that of the MEF cells (Figure 5). H_2O_2, which did not induce HSP70 in MEF cells induced the *hsp*70.1 gene expression in the M-1 cell line. The *hsp*70.1 transcript was detected in all applied stressors. The result of immunoblot assay in M-1 cells was consistent with that of Northern analysis (data not shown). From our results in MEF and M-1 cells, we concluded that the two isoforms of *hsp*70 are differentially induced in different cells and by different stressors.

Discussion

All organisms face environmental and pathophysiological stresses, and must develop appropriate protective mechanisms. One of the most well known protective mechanisms is to rescue cells through the induction of stress response proteins (Hightower, 1991; Flanagan *et al.,*1995; Schliess *et al.,* 1999). Inducible HSP70 has been known as a molecule that has a pivotal role in the protection of cells from various stresses. It was initially observed as the most prominently induced protein in cells subjected to elevated temperature (Lindquist and Craig, 1988). HSP70 synthesis has been identified in response to a wide range of chemicals and biological signals, including heavy metals, amino acid analogues, inhibitors

of energy metabolism, UV- and γ-irradiation, oxidative stress, viral and bacterial infections, and magnetic fields (Morimoto *et al.,* 1990; Sierra-River *et al.,* 1993; Hatayama *et al.,* 1993; Abe *et al.,* 1995; Goodman and Blank, 1998; Wagner *et al.,* 1999). These observations suggest that HSP70 is involved in a general cellular defense mechanism. Recently, its suspected role was strongly supported by the reports that HSP70 acts as an inhibitor of apoptosis (Samali and Cotter, 1996; Ahn *et al.,* 1999; Beere *et al.,* 2000; Li *et al.,* 2000; Saleh *et al.,* 2000). Therefore, HSP70 might be a useful target molecule for the therapeutic treatment of apoptosis-related diseases.

The fact that inducible HSP70 is encoded from both *hsp*70.1 and *hsp*70.3 genes, which show high similarity in their coding sequences and a linked tandem array with MHC, caused us to question why many higher organisms have almost identical genes in duplicate. The fact that the two inducible *hsp*70 genes differ from each other in the 5- and 3-untranslated regions (Walter *et al.,* 1994), suggests that they might be under differential regulation. However, the data indicating that *hsp*70.1 and *hsp*70.3 can be differentially expressed are very limited to date. Therefore, it remains to be determined whether the expression of *hsp*70.1 and *hsp*70.3 occurs concordantly (Dix *et al.,* 1998; Akçetin *et al.,* 1999). This may be determined by measuring mRNA expression levels with probes specific for each gene, and the result of present study using *hsp*70.1 knockout mice could give a more convincing conclusion.

In this study, we observed the expression pattern of HSP70 by treating MEF from *hsp*70.1-/- mice with various *hsp*70-inducible agents. No obvious difference in the expression patterns of the *hsp*70.1 and *hsp*70.3 genes was observed when MEF cells were treated with heat shock, heavy metal ions, or amino acid analogue. However, hyperosmolarity induced only *hsp*70.1 expression. In addition to this, the basal level of HSP70 in untreated MEF cells depended on the existence of the *hsp*70.1 gene, and *hsp*70.1 was found to respond to a broader range of stresses than *hsp*70.3. We next observed the expression pattern of *hsp*70 genes in the M-1 cell line. Hyperosmotic and oxidative stresses induced only *hsp*70.1 in the M-1 cell line. Our result is supported by reports that the *hsp*70.1 gene mediates the major response of heat shock protein induction after severe injury in the rat kidney (Akçetin *et al.,* 1999) and infarction volume after focal cerebral ischemia was increased in *hsp*70.1-/- mice (Lee *et al.,* 2001). The result of hyperosmotic stress in M-1 cells was consistant with that in MEF cells. Whereas, oxidative stress did not induce both *hsp*70 genes in MEF cells, but induced the *hsp*70.1 gene in the M-1 cell line. These results show that some stresses specifically induce one of the *hsp*70 genes, and some induce the *hsp*70 genes in a cell-type specific manner. Our study provides strong evidence that *hsp*70.1 and *hsp*70.3 genes respond differentially to different stress types.

It is known that HSP70 accumulation in cells, which are exposed to stress, depends on heat-shock factor (HSF) activation (Abravaya *et al.,* 1991; Morimoto, 1992). Stress-induced rapid activation of HSF is a very common phenomenon. Moreover, it has been suggested that inducible HSP70 synthesis appears to be regulated by an alternative factor, constitutive heat shock element-binding factor (CHBF) (Liu *et al.,* 1995). There is a possibility that different regulatory factors might be involved in specific gene activation events for each inducible *hsp*70 gene, because two *hsp*70 genes have different promoter regions. This is supported by a report that a mutant of regulatory factor showed differential activation of *hsp*70 in response to various stresses (de La Serna *et al.,* 2000). How *hsp*70 genes are differentially regulated needs further investigation and currently we are experimenting with promoters of each *hsp*70 gene to identify the differential regulatory mechanism.

Acknowledgements

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