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# Expression of the Heat Shock Proteins in HeLa and Fish CHSE-214 Cells Exposed to Heat Shock

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**In this study, we examined the expression of heat shock proteins (HSPs) in fish cell line CHSE-214 and human HeLa cells exposed to heat shock. In fish CHSE-214 cells HSP70 was the major polypeptide induced by an elevated temperature or an amino acid analog, while in HeLa cells HSP90 as well as HSP70 were prominently enhanced in response to these stresses. Pretreatment of actinomycin D prior to heat shock completely inhibited the induction of fish HSP70, indicating the transcriptional regulation of fish HSP70 gene expression. In HeLa and CHSE-214 cells either recovering from heat shock or experiencing prolonged heat shock, attenuation in the HSP90 and HSP70 induction occurred but both induction and repression of HSP70 synthesis appear to precede those of HSP90. Moreover, attenuation did not occur in the syntheses of 40 kDa and 42 kDa proteins which were only induced in CHSE-214 cells. The enhanced syntheses of these two proteins continued as long as CHSE-214 cells were given heat shock. These results suggest that down-regulation of HSP syntheses during prolonged heat shock may be controlled by several different, as yet undefined, mechanisms.**

**KEY WORDS: Heat Shock Proteins, HeLa Cells, Fish CHSE-214 Cells, Attenuation**

The heat shock or stress response refers to the reaction of living cells to adverse environmental conditions (Welch *et al.*, 1991). The stress response results in the rapid and coordinated induction of a group of proteins referred to as the stress proteins and the concomitant reduction of normal cellular proteins. The stress proteins can be divided into two groups of families: the heat shock proteins (HSPs) that are mainly induced by exposure of cells to elevated growth temperatures, amino acid analogs, or various heavy metals (Klann and Shelton, 1989; Schlesinger, 1990) and the glucose-regulated proteins (GRPs) whose levels are enhanced by glucose starvation, inhibition of protein glycosylation or perturbation in intracellular $Ca^{2+}$ homeostasis (Kang *et al.*, 1995; Subject *et al.*, 1986; Welch *et al.*, 1990). HSPs family includes HSP60, HSP70 and HSP90 that are present in the cytosol and/or nuclei, while GRPs family consists of GRP78/BiP and GRP94 in the endoplasmic reticulum. The HSPs and GRPs are co-related to each other in respect to their amino acid sequences and cellular functions. For example, HSP70 and GRP78/BiP have a sequence homology and function as molecular chaperones which regulate *in vivo* protein folding and/or assembly (Beckmann *et al.*, 1990; Chirico *et al.*, 1988; Deshaies, 1988).

The heat shock response is highly conserved during evolution and most of the HSPs are also constitutively synthesized in considerable amounts even in the unstressed normal cells (Hunt and

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Morimoto, 1985; Thomas *et al.*, 1982; Welch *et al.*, 1991). These facts lead to a suggestion that the expression of HSPs must be essential for the survival of the unstressed cells as well as of the stressed cells. One of the major effects of heat shock is an unfolding or incomplete folding of proteins appeared early in heat-shocked cells (Hightower, 1980). HSPs are proposed to play an role (s) in protection against these stresses, accelerating the recovery of cellular proteins.

The heat shock response is mediated by heat shock transcription factor (HSF), which binds to regulatory heat shock elements (HSEs), present upstream of all heat shock genes (Abravaya *et al.*, 1992). In higher eukaryotes, HSE is composed of contiguous repeats of the 5-bp sequence NGAAN; three NGAAN repeats are required for high affinity interaction with HSF (Xiao and Lis, 1988; Perisic *et al.*, 1989). DNA binding activity of HSF is controlled by transition from monomeric (unactivated) to trimeric (activated) state, which is induced by heat stress (Sarge *et al.*, 1993; Westwood and Wu, 1993). DNA interaction affinity of monomeric HSF is relatively low ($Kd=10^{-7}$-$10^{-8}$ M) and trimerization increases the affinity for HSE (Kim *et al.*, 1994).

In higher eukaryotes such as human HeLa and K562 cells, transcriptional activation of HSP genes is transient and occurs only during the initial phase of heat shock, following which attenuation of heat shock gene transcription is observed (Abravaya *et al.*, 1991). HSPs have been suggested to participate in the autoregulation of the heat shock response. Under normal growth conditions, HSPs, in particular HSP70 and HSP90, bind to and stabilize the latent HSF monomer, which suppreses the HSF activity. During heat shock, high levels of stress-induced misfolded polypeptides are accumulated and compete with HSF for binding to HSPs, thereby resulting in the dissociation of HSF-HSP complexes and trimerization of HSF. DNA-bound trimeric HSF then enhances the expression of HSP genes until HSP levels are sufficiently high to result in the reassociation of HSPs and HSF and the reestablishment of the repressed state of HSF.

The heat shock response has been reported in a wide range of organisms, including bacteria (Neidhardt and VanBogelen, 1987), plant (Barnett *et al.*, 1980), insect (Ashburner and Bonner, 1979), and a number of mammalian cell lines (Welch, 1986). However, relatively little is known about the heat shock response in poikilothermic animals which may differently adapt to heat shock or regulate the HSP expression (Misra *et al.*, 1989). Particularly, aquatic organisms are confronted with a variety of environmental stresses including fluctuation in temperature but the heat shock response of these organisms is not well characterized. In this study, we examined the expression of HSPs in fish cell line CHSE-214 exposed to heat shock and compared with that of human HeLa cells.

## Materials and Methods

### Cell culture and heat shock and chemical treatments

Chinook salmon embryonic cells (CHSE-214) were grown at 18°C in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HeLa cells were cultured in DMEM supplemented with 10% calf serum (CS) and 1% penicillin/streptomycin at 37°C in an incubator with 5% $CO_2$. For heat shock treatment, subconfluent CHSE-214 and HeLa cells were exposed to 24°C and 43°C, respectively, for several hours as specified in figure legends. In recovery experiments, 2 h heat-shocked cells were placed to normal growth temperatures. Some cells were treated with 5 mM 5-azacytidine, 300 nM thapsigargin or 0.1 μg/ml tunicamycin to induce the stress response.

### Metabolic labeling and SDS-polyacrylamide gel electrophoresis

For metabolic labeling, cultured CHSE-214 and HeLa cells were washed in methionine-free DMEM and proteins were then labeled with [$^{35}$S] methionine (30 μCi/ml: Amersham Corp.) in methionine-free DMEM for the last 1 h of incubation unless otherwise stated. Cells were washed three times in cold phosphate-buffer saline (PBS) and harvested in 2 × Laemmli sample

buffer (LSB). To prepare sample for sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), cellular proteins in LSB were heated at 100°C for 3 min. The quantitation of labeled proteins was determined by Liquid Scintillation Counter (LKB). SDS-PAGE was carried out on 12.5% polyacrylamide gel as described by Laemmli (1970). After the electrophoresis, gels were stained and then dehydrated with dimethyl sulfoxide (DMSO) and amplified with 15% PPO in DMSO. Fluorography was carried out at −80°C using X-ray film. Molecular weight calibration was made using protein markers. The quantification of the autoradiogram was carried out using a densitometric scanner (TLC CS-930, Shimadzu Co., Japan).

### Western blot analysis

Western blotting was performed as described in Towbin *et al.* (1979). The electrophoresed gels were transferred to nitrocellulose papers and membranes were then blocked with 5% nonfat milk in TBS-T for 1 h at room temperature. Membranes were then incubated with either anti-HSP90 MoAb (C112, produced in our laboratory, Kong *et al.*, 1995), anti-HSP72 MoAb (C92, StressGen, Victoria, British Columbia, Canada), anti-HSP72/73 MoAb (N27, a gift from Dr. W. J. Welch, Univ. of California, San Francisco, CA) for 1-2 h at room temperature. After washing in TBS-T three times, the blot was incubated with peroxidase-conjugated secondary antibody and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp.).

### Northern blot analysis

Total RNA was isolated using TRI reagent according to manufacturer's protocol (Molecular Research Center Inc., Cincinnati, OH) and electrophoresed on a denaturing gel (1% agarose, 2.2M formaldehyde). For Northern blotting (Maniatis *et al.*, 1989), RNA was transferred onto nylon membrane (Zeta-Probe blotting membrane, Bio Rad, CA) and hybridized with either human HSP70A (a gift from Dr. R. I. Morimoto, Northwestern Univ., IL) or HSP70B (StressGen, Victoria, British Columbia, Canada) cDNA probes which had been labeled with [$\alpha$-$^{32}$P]dCTP (Amersham Corp.) by random priming. The filter was then exposed to X-ray film for 2-5 days.

## Results

As shown in Fig. 1 and as others have shown previously, exposure of HeLa cells to either 5-azacytidine or an elevated growth temperature (43°C) caused the great induction of stress proteins. The syntheses of HSP90 and HSP70 were prominently enhanced by these stresses. In addition to HSP90 and HSP70, 5-azacytidine treatment also induced the synthesis of GRP78/BiP homologous to HSP70 and presented exclusively in the endoplasmic reticulum. Further, treatment of tunicamycin, a protein N-glycosylation inhibitor, or thapsigargin, an endomembrane $Ca^{2+}$-ATPase inhibitor, increased the synthesis of GRP78/BiP and, to a less extent, that of GRP94.

In order to examine the syntheses of fish stress proteins, CHSE-214 cells were exposed to an

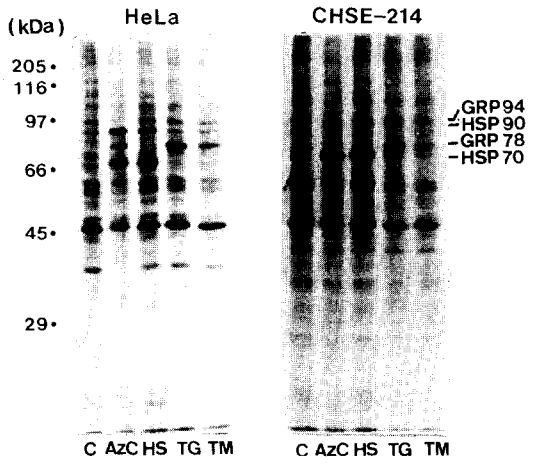


**Fig. 1.** Induction of stress proteins in HeLa and CHSE-214 cells. HeLa and CHSE-214 cells were exposed to 43°C and 24°C, respectively, for 2 h (lane HS), 5 mM 5-azacytidine for 7 h (lane Azc), 300 nM thapsigargin for 7 h (lane TG) or 0.1 $\mu$g/ml tunicamycin for 5 h (lane TM). The cells were labeled in [$^{35}$S]methionine for the last 1 h and the cellular proteins were analyzed by SDS-PAGE and fluorography. Lane C represents the control.

elevated growth temperature (24°C) or stressful agents including 5-azacytidine, tunicamycin and thapsigargin. In CHSE-214 cells, HSP70 was the major polypeptide induced in response to 5-azacytidine and heat shock, while HSP90 was also induced but at a much lower level (Fig. 1). The syntheses of GRP78/BiP and GRP94 were also enhanced by tunicamycin or thapsigargin (Fig. 1). Pretreatment of the transcriptional inhibitor actinomycin D (20 μg/ml) for 30 min prior to heat shock (24°C) completely inhibited the induction of HSP70, indicating that the HSP70 induction of fish CHSE-214 cell is regulated at the transcriptional level (Fig. 2).

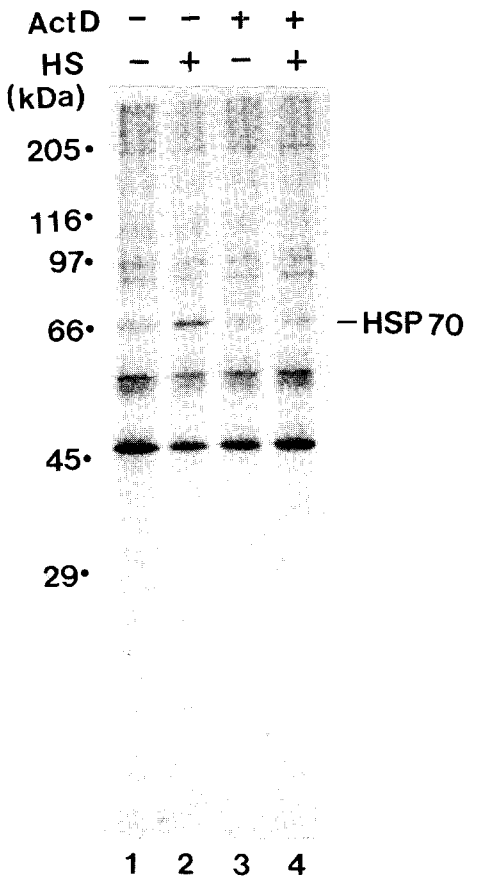


**Fig. 2.** Effect of actinomycin D on the syntheses of fish HSPs. CHSE-214 cells were incubated in the absence (lanes 1-2) or presence (lanes 3-4) of actinomycin D (20 μg/ml) for 30 min and then incubated at 18°C (lanes 1 and 3) or 24°C (lanes 2 and 4). The cells were labeled in [$^{35}$S]methionine (30 μCi/ml) for the last 1 h and the cellular proteins were analyzed by SDS-PAGE and fluorography.

The protein synthesis patterns in HeLa and CHSE-214 cells recovering from heat shock were compared. As shown in Fig. 3, the elevated synthesis of HSP70 was observed in both HeLa and CHSE-214 cells during 2 h heat shock (43°C and 24°C, respectively) and maintained by 3 h recovery at 37°C (in HeLa cells) or 6 h recovery at 18°C (in CHSE-214 cells) and thereafter rapidly diminished, its synthesis returning to translation patterns similar to that of the normal cells. On the other hand, the great induction in HSP90 synthesis was observed in HeLa and CHSE-214 cells during 3 h recovery at normal growth temperatures, although slight increase in HSP90 synthesis was also detected during heat shock treatment. The synthesis of HSP90 persisted at the elevated level until 6-9 h recovery and thereafter gradually reduced, its synthesis with returning to the basal level. Thus, both induction and repression of HSP70 synthesis appear to precede those of HSP90 in HeLa and CHSE-214 cells recovering from heat shock.

Furthermore, the protein syntheses in HeLa and

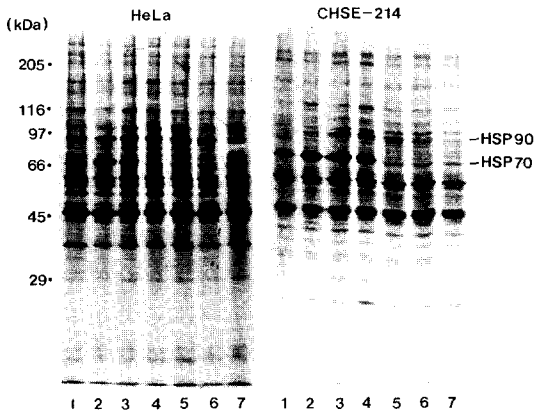


**Fig. 3.** Syntheses of HSPs in HeLa and CHSE-214 cells recovering from heat shock. HeLa and CHSE-214 cells were incubated for 2 h at either 43°C or 24°C, respectively, and then recovered to normal growth temperature 37°C or 18°C for up to 15 h. The cells were labeled in [$^{35}$S]methionine (30 μCi/ml) for the last 2 h and the cellular proteins were analyzed by SDS-PAGE and fluorography. Lane 1, control; lane 2, heat shocked for 2 h; lanes 3-7, recovered from heat shock for 3, 6, 9, 12 and 15 h, respectively.

CHSE-214 cells exposed to prolonged heat shock were also examined. In HeLa cells, a very rapid induction of HSP70 synthesis was observed during the initial phase (3 h) of heat shock (43°C) and the elevated synthesis of HSP70 continued for additional 3 h and then declined to control level despite the prolonged heat shock exposure (Fig. 4), similarly to the results obtained in recovery experiments. Such an attenuation was also observed in HSP90 synthesis and the induction and repression of HSP90 synthesis occurred more slowly that those of HSP70.

The expression of HSP70 gene during continued heat shock was examined by Northern blot analysis using cDNA of human HSP70A and HSP70B. HSP70A gene codes HSP72 and HSP70B gene shows high homology to HSP70A except 5′ terminus (Hunt and Morimoto, 1985; Voellmy *et al.*, 1985). As shown in Fig. 5, HSP70A and HSP70B gene expression markedly increased by 1 h heat shock treatment and then gradually attenuated during prolonged heat shock, although HSP70A mRNA level persisted at the elevated level longer than that of HSP70B.

Similar attenuation phenomenon was also observed in syntheses of HSP90 and HSP70 in CHSE-214 cells subjected to prolonged heat shock. As shown in Fig. 6, a significant induction of HSP70 synthesis was observed at 3 h following temperature upshift (24°C) and maintained until 6-12 h of heat shock and then reduced to the control level by prolonged heat shock for 18 h. As the cells were given more prolonged heat shock for 30 h, HSP70 synthesis resumed to increase gradually. The increased synthesis of HSP90 was observed at 6-9 h following heat shock and then declined during continued exposure to heat shock. Thus, as in HeLa cells, HSP70 and HSP90 synthesis attenuated despite the prolonged exposure to an elevated temperature and the induction and repression of HSP70 occured more rapidly that those of HSP90. Interestingly, two polypeptides having molecular weights of 40kDa and 42kDa were also induced by heat shock, but the induction kinetics of the 40kDa and 42kDa proteins were different from those of HSP90 and HSP70. The enhanced syntheses of these two

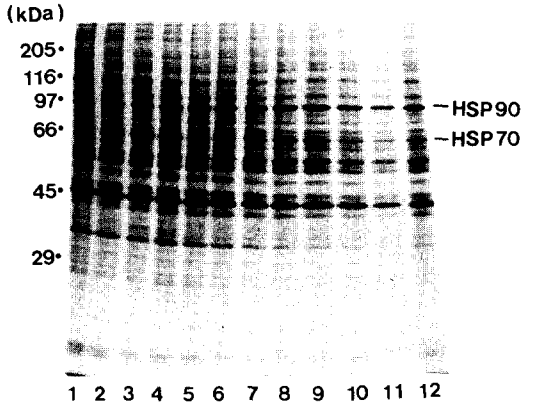


**Fig. 4.** Protein synthesis pattern in HeLa cells exposed to prolonged heat shock. HeLa cells were incubated at 37°C (lane 1) or 43°C for 1, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30 h (lanes 2-12, respectively). To label cellular proteins, [$^{35}$S]methionine (30 $\mu$Ci/ml) was added for the last 1 h and the labeled proteins were analyzed by SDS-PAGE and fluorography.

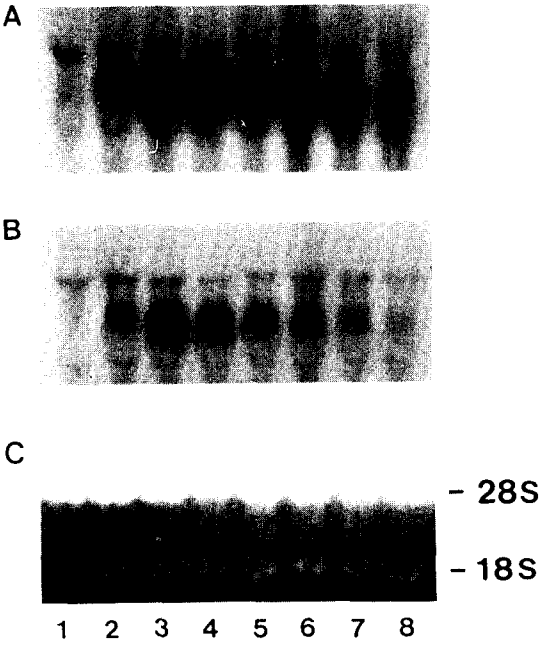


**Fig. 5.** Northern blot analysis of HSP70 expression in HeLa cells exposed to prolonged heat shock. HeLa cells were incubated at 43°C for 1, 3, 6, 12, 18, 24, 30 h (lanes 2-8, respectively). RNA was isolated and analyzed by agarose gel electrophoresis and blotted with cDNA probes for HSP70A (A) or HSP70B (B). Ethidium bromide-stained RNA pattern is shown in panel C.

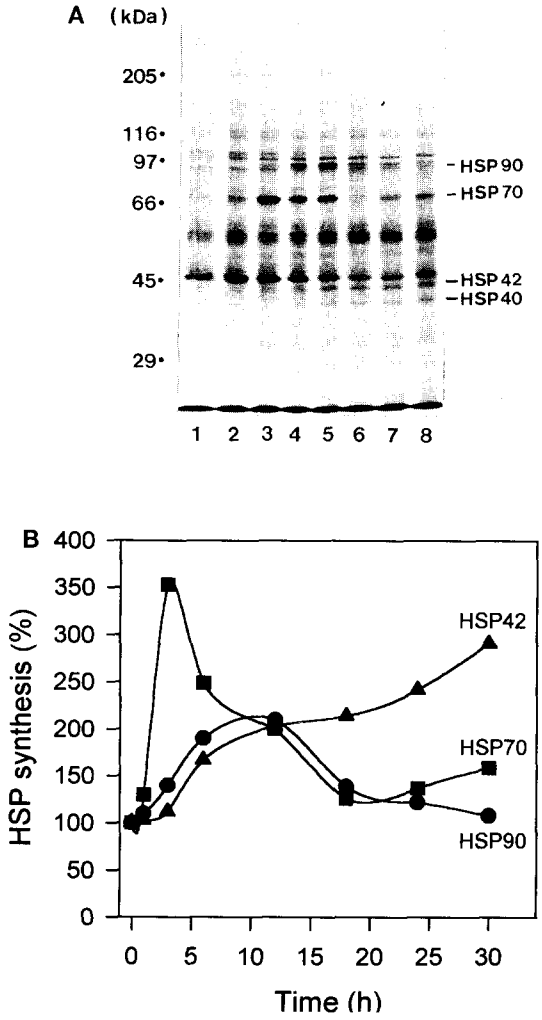


**Fig. 6.** Synthesis of heat shock proteins in CHSE-214 cells exposed to prolonged heat shock. CHSE-214 cells were incubated at either 18°C (lane 1) or 24°C for 1, 3, 6, 12, 18, 24, and 30 h (lanes 2-8). To label cellular proteins, [$^{35}$S]methionine (30 $\mu$Ci/ml) was added for the last 1 h and the labeled proteins were analyzed either by SDS-PAGE and fluorography (panel A) and the change in HSP synthesis was quantitated by densitometric scanning (panel B).

proteins continued as long as heat shock was given to CHSE-214 cells (Fig. 6).

The relative protein levels of HSP90 and HSP70 in CHSE-214 cells experiencing prolonged heat shock were examined by Western blotting using monoclonal anti-HSP90 antibodies

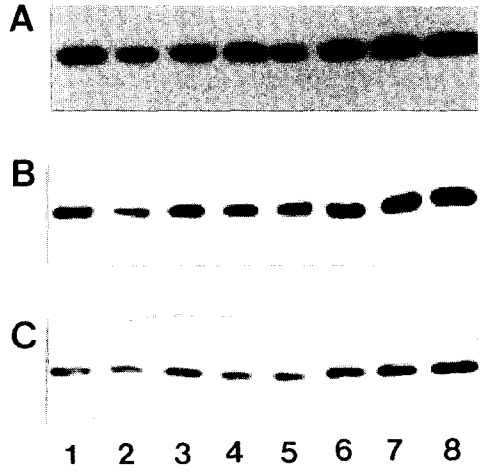


**Fig. 7.** Changes in HSP70 and HSP90 levels in CHSE-214 cells exposed to prolonged heat shock. CHSE-214 cells were incubated at either 18°C (lane 1) or 24°C for 1, 3, 6, 12, 18, 24, and 30 h (lanes 2-8) and changes in HSP70 and HSP90 levels were analyzed by Western blotting with anti-HSP90 (C112, panel A), anti-HSP72/73 (N27, panel B) or anti-HSP72 (C92, panel C).

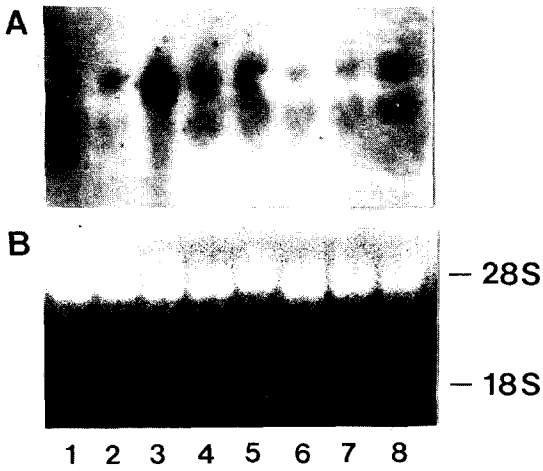


**Fig. 8.** Northern blot analysis of HSP70 expression in CHSE-214 cell exposed prolonged heat shock. CHSE-214 cells were incubated at either 18°C (lane 1) or 24°C for 1, 3, 6, 12, 18, 24 and 30 h (lanes 2-8, respectively). RNA was isolated and analyzed by agarose gel electrophoresis and blotted with cDNA probe for HSP70A (A). Ethidium bromide-stained RNA pattern is shown in panel B.

(C112) or anti-HSP70 antibodies (N27 and C92). As shown in Fig. 7, exposure of CHSE-214 cells to prolonged heat shock gradually increased the levels of both HSP90 and HSP70.

The expression of HSP70 gene during prolonged heat shock was also examined by Northern blot analysis using cDNA of human HSP70A and HSP70B. HSP70 mRNA of fish CHSE-214 cells was successfully hybridized with human HSP70A cDNA but not with HSP70B cDNA (data not shown). As shown in Fig. 8, HSP70A gene expression markedly increased during the initial phase of heat shock and then attenuated by prolonged heat shock exposure, consistent with observation of HSP70 protein synthesis.

## Discussion

All organisms respond to elevations of 5°C or more above their normal physiological temperatures with the rapid and preferential syntheses of the HSPs (Welch *et al.*, 1991). The heat shock response is also elicited in response to a wide variety of environmental insults such as amino acid analogs and heavy metals (Klann *et al.*, 1989; Schlesinger *et al.*, 1990). Inhibition of protein glycosylation or perturbation of $Ca^{2+}$ homeostasis can induce the syntheses of the GRPs (Kang *et al.*, 1995; Subject *et al.*, 1986), which are homologous to the HSPs but mostly reside in the endoplasmic reticulum. In this study, we demonstrate that HSP70 synthesis is enhanced in fish cell line CHSE-214 subjected to an elevated growth temperature or 5-azacytidine (an amino acid analog), while those of GRP78/BiP and GRP94 are induced by tunicamycin (a N-glycosylation inhibitor) and thapsigargin (a $Ca^{2+}$ ATPase inhibitor) (Fig. 1). In addition, two proteins of 40kDa and 42kDa were also induced in CHSE-214 cells experiencing prolonged heat shock (at 24°C for 30 h) (Fig. 6). Induction of these proteins was not detected in either HeLa cells exposed to continued heat shock or in HeLa and CHSE-214 cells heat shock for a short period or recovering from heat shock. These proteins may be fish-specific HSPs that are inducible only by continued heat shock. Another feature of fish heat shock response is that HSP70 is a major stress-inducible HSP: HSP90 is increased at a much lower level compared to HSP70. Neither 5-azacytidine known to act as a very potential inducer of heat shock response in cells examined so far (Nover, 1984), nor prolonged/higher heat shock treatment did induce the synthesis of HSP90. Fish cells may evolve the more simple and primitive stress response than homeothermic cells.

The heat shock gene expression is regulated mostly at the transcriptional level (Hunt and Morimoto, 1985; Wu *et al.*, 1987), though the stability or translational efficiency of heat shock induced mRNAs was also demonstrated to increase in stressed cells (Lindquist, 1980). Fish HSP70 expression appears to be regulated at the transcriptional level (Fig. 2).

As expected, in HeLa and fish CHSE-214 cells, the expression of HSP70 and HSP90 decreased upon returning to pre-heat shock temperature (recovery) (Fig. 3). Moreover, attenuation of the heat shock response also occurred in HeLa and CHSE-214 cells during prolonged exposure to elevated temperatures (Figs. 4-8), though relative levels of HSPs gradually increased by continued heat shock (Fig. 7). Attenuation of the heat shock response occurs in a variety of eukaryotic cells and has been proposed to be mediated by HSF, activated to trimeric state by heat stress and which binds to HSE located in the promoters of heat-induced genes (Green *et al.*, 1995). In the normal cells, HSF exists complexed with HSPs, especially HSP70 and HSP90, which stabilize HSF in an inactive monomeric state. Upon heat shock, the factor is dissociated from HSPs and can activate the heat shock-reponsive genes (Abravaya *et al.*, 1992). As the levels of HSPs increase in proportion to heat shock, HSF and HSPs are reassociated, thereby causing attenuation of heat shock gene transcription.

Although in both HeLa and CHSE-214 cells either recovering from heat shock or experiencing prolonged heat shock, attenuation in the expression of HSP90 and HSP70 occurs as described by other investigators (Abravaya *et al.*, 1991), the attenuation kinetics of HSP70 and HSP90 were different: both induction and

repression of HSP70 expression appear to precede those of HSP90. As all HSP genes contain similar HSEs accessible by activated HSF, the induction and repression of HSP90 and HSP70 must occur at the same time. Moreover, the attenation cannot be applied to the syntheses of 40kDa and 42kDa proteins that continue to be induced in CHSE-214 cells as long as the cells were given heat shock. Therefore, another regulatory mechanism, as yet undefined, may exist by which the expression of HSP70 and HSP90 is differently regulated. At present, the possible regulatory mechanism underlying this differential attentuation between HSPs is under investigation.

## Acknowledgements

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## 어류 CHSE-214와 인간 HeLa 세포에서의 열충격에 의한 Heat Shock Protein의 발현

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어류세포인 CHSE-214와 인간의 HeLa 세포에서 열충격을 가한 후 Heat Shock Proteins의 발현을 비교, 조사하였다. 어류 CHSE-214 세포에서는 높은 성장온도와 아미노산 유사체 처리에 의해서 HSP70의 발현이 현저히 증가하였으나 HeLa 세포에서는 HSP70 뿐만아니라 HSP90의 발현도 상당량 증가하였다. 열충격을 가하기 전에 actinomycin D를 처리하였을 경우 어류의 HSP70 합성이 억제되는 것으로 보아 HSP70 유전자 발현은 전사 수준에서 조절됨을 알 수 있다. HeLa와 CHSE-214 세포에서 열충격 후 정상적인 성장온도로 회복시켰을 때나 계속해서 열충격을 가할 때 HSP70과 HSP90의 발현이 증가하다가 다시 감소하는 attenuation 현상이 관찰되었으며 HSP70 합성의 유도나 억제가 HSP70이 HSP90의 경우보다 빠르게 일어나는 것으로 나타났다. 또한 CHSE-214 세포에서만 유도되는 40kDa, 42kDa 단백질의 합성에서는 attenuation 현상이 일어나지않고 열충격이 가해지는 한 계속해서 합성이 증가하는 양상을 보였다. 이러한 결과들로 미루어보아 열충격이 계속될 때 HSP 합성의 down-regulation은 아직 밝혀지지 않은 여러 기작들에 의해서 조절받고 있는 것으로 생각되어진다.