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Expression Patterns of Heat Shock Proteins in Primary Cultured Hepatocytes from Flounder (*Paralichthys olivaceus*)

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We examined the expression patterns of heat shock proteins in primary cultured hepatocytes from flounder (*Paralichthys olivaceus*) exposed to heat shock. The expression of hsp90, hsp 70, hsp40, hsp30, and hsp27 was induced and major polypeptides were hsp70, hsp30 and hsp 27. Northern blot analysis showed that expression of hsp70 was inhibited by transcriptional inhibitor actinomycin D, suggesting that expression of hsp70 gene is regulated at the transcriptional level. Prolonged exposure of cells to an elevated incubation temperature (30℃) induced the transient synthesis of hsp90, hsp70, hsp40, and hsp30 whereas maintenance of cells at a slightly higher incubation temperature (32℃) induced the continuous syntheses of these hsps. When cells were incubated at a higher temperatures (35℃ or 37℃), the synthesis of hsps was almost similar to that of hsps in cells exposed to 32℃ except for the induction of hsp27 synthesis. These results that temperature and incubation time for optimum expression of each hsp during prolonged heat shock are different.

Key words: heat shock proteins, flounder, *Paralichthys olivaceus*, hepatocyte, Northern blot analysis

Introduction

All prokaryotic and eukaryotic cells exhibit increased synthesis of a set of proteins referred to as stress or heat shock proteins (hsps) in respond to a sudden increase in physiological temperature (Schlesinger et al, 1982). Hsps are also induced by other physiological stresses such as amino acid analogues, heavy metals, and sodium arsenite (Hightower, 1980; Levinson et al, 1980; Kothary and Candido, 1982; Misra et al, 1989). The major classes of hsps are distinguished by their molecular weights such as hsp90, hsp70, hsp60, and low molecular weight hsps (Ashburner and Bonner, 1979). The synthesis of hsp70 is greatly induced by heat shock and hsp70 is highly conserved in organisms from bacteria to human (Schlesinger et al, 1982). The heat shock response has been reported in a wide range of organisms, including bacteria (Neidhardt and VanBogelen, 1987), *Drosophila* (Ashburner and Bonner, 1979), plant (Barnett et al, 1980), a number of mammalian cell lines (Craig, 1985; Lindquist, 19 86), a few freshwater fish cells (Heikkila et al, 1982; Mosser et al, 1986; Koban et al, 1987; Chen et al, 1988; Mitani et al, 1989; Kong et al, 1996), and oyster haemocytes (Tirard et al, 1995).

Hsps function both in stressed and in non-stressed cells. In stressed cells, hsps serve to protect proteins from denaturation and appear to be involved in the development of thermotolerance (Li and Werb, 1982; Chen et al, 1988; Hahn and Li, 1990). In non-stressed cells, hsps play a vital role in basic cellular processes, acting as molecular chaperones directing the folding, translocation and assembly of cellular proteins (Chirico et al, 1988; Beckman et al, 1990; Geothing and Sambrook, 19 92). These results suggest that the expression of hsps is essential for the survival of both stressed and non-stressed cells. The expression of hsps has been shown to be regulated by both transcriptional and translational levels (Lindquist, 1980a; Mosser et al, 1988)

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Both primary cultured cells and cell lines derived from fish are able to grow within a wide range of temperature (Wolf and Quimy, 1962; Mosser et al, 1986). These characteristics make fish a good system to study the heat shock response and thermal adaptation process. The seawater fish are confronted with a variety of environmental stresses including fluctuation in temperature which is a critical factor for aquatic poikilotherms. However, the heat shock response in seawarter fish cells is not well characterized. Flounder, one of the poikilothermic fish, is able to tolerate rapid and large temperature changes. It can survive from 10℃ water in winter to about 28℃ in summer in all coasts of Korea, therefore they should have protective mechanisms to such a great thermal change. In this study, we examined the expression patterns of hsps in primary cultured hepatocytes isolated from flounder exposed to heat shock.

Materials and Methods

Fish

Flounders used in this study were obtained from Koje Hatchery of National Fisheries Research and Development Institute and acclimated at 18 ± 1℃ under a natural photoperiod for at least 30 days before use. We used flounders with about 15-18 cm in body length.

Preparation of hepatocytes and cell culture

Hepatocytes were isolated by a modification of two-step hepatic perfusion procedure described by Seglen (1977). After flounder was anesthetized with 0.01% p-aminobenzoate, the liver was exposed and perfused with Ca^{2+}-free buffer solution (110 mM NaCl, 4 mM KCl, 1.25 mM, 25 mM $NaHCO_3$, 0.5 mM EDTA, 5 mM D-glucose, and 0.2 mg/mℓ heparin) for 10 min and then perfused with Ca^{2+}-collagenase buffer (110 mM NaCl, 4 mM KCl, 1.25 mM, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 5 mM D-glucose, and 0.05-0.1% collagenase). When the liver was thoroughly blanched, swollen, and malleable, it was removed and chopped apart with forceps. The tissues were further digested in collagenase perfusion buffer for 15 min in slowly rotating shaker. Dispersed liver tissues were removed once through a tissue sieve and centrifuged at 500 rpm for 4 min. The precipitates were resuspended in Leibovitz's L-15 medium and washed three times by centrifugation. Hepatocytes were resuspended in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were seeded onto tissue culture flasks and were grown routinely at 18 ± 0.1℃ in regulated air-incubators. During perfusion and isolation of cells, all buffers and culture media were carefully maintained at 18℃.

Heat shock, metabolic labeling, and sodium dodecyl sulfate-polyacryamide gel electrophresis

Hepatocytes were allowed to occur the attachment at 18℃ for 24h. For measuring temperature profile against heat shock, the cells growing on 35-mm dishes were incubated at 21, 24, 27, 30, 32, and 35℃ for 2h. For prolonged heat shock, cells were incubated at 30, 32, 35, and 37℃ for various periods of incubation time as indicated in figure legends (Fig. 4, 5, and 6). The transcriptional inhibitor actinomycin D (20 μg/mℓ) was added to hepatocytes for 30 min prior to heat shock and was present throughout the experiments as described in legend of the figure 3. For metabolic labeling, the culture medium was removed and cells were washed with methionine-free Dulbecco's modified Eagles medium (DMEM) and proteins were labeled with [^{35}S]-methionine (62 μCi/mℓ) for 2h. Then cells were washed three times with phosphate-buffered saline (PBS) and harvested in 2 × Laemmli sample buffer (LSB). To separate cellular protiens in LSB by SDS-PAGE, samples were boiled at 100℃ for 3 min. The quantification of labeled proteins was determined by Liquid Scintillation Counter. Equivalent amounts of radioactivity were loaded for each sample. SDS-PAGE was carried out on 12.5% polyacrylamide gel as described by Laemmli (1970). Protein concentrations were determined using the bicinconinic acid (BCA) assay (Sigma) with bovine serum albumin (BSA) as a standard. After electrophoresis, gels were stained with Coomassie Blue and then dehydrated with dimethyl sulfoxide (DMSO), and amplified with 15% 2,5-diphenyl-oxazole (PPO) in DMSO (Laskey and Mills, 1975). Fluorography was carried out at −80℃ using Kodak XAR-5 X-ray film. The quantification of each hsp was carried out using a densitometric scanner (Bio-Rad).

Northern blot analysis

Total RNA was isolated from the flounder hepatocytes using Trizol RNA isolation reagent (GIBCO/BRL) according to the manufacturer's instructions (Chomczynski et al., 1987). cDNA was

prepared from total RNA by oligo (dT)-primed reverse transcription according to the manufacturer's instructions (GIBCO/BRL, USA) and used as template for PCR amplification. Two pairs of primers derived from the nucleotide sequence of flounder *hsp70*-related cDNA (accession number AF053059) (Kim et al., 1999) were used to amplify a highly conserved portion (the forward primer 5′-GATGAAGCTGTGGCCTACGGAG-3′ and the reverse primer 5′-GATGCCGTTGGCATCAATATC-3′). The PCR cycle consisted of 1 min denaturation at 94℃, 1 min annealing at 55℃, and 1 min extension at 72℃ for 35 cycles. Expected PCR products were cloned into the TA cloning vector pCR2.1 (Invitrogen), sequenced, and used as probe. Total RNA (25 μg) from hepatocytes incubated in the absence or presence of actinomycin D were denatured in formamide and formaldehyde at 65℃ for 5 min and electrophoresed in an agarose gel containing formaldehyde (Sambrook et al., 1989). RNA was then transferred onto a nylon membranes (Bio-Rad) and fixed to the membrane using the UV cross linker. cDNA probe was labeled by using [α-^{32}P] dCTP with random priming kit (Amersham) and were hybridized to the membranes in hybridization buffer (50% formamide, 0.25 M $NaHPO_4$, 7% SDS and 1mM EDTA) at 42℃ for 14hr. Membranes were washed in 2 × SSC-0.1% SDS at room temperature (RT), then in 0.1 × SSC and 0.1% SDS at 65℃ for 15 min each and visualized by autoradiography with Kodak XR5 film at −80℃.

Western blot analysis

Western blot analysis was performed as described by Towbin et al. (1979). The electrophoresed gels were transferred to nitrocellulose papers and the membranes were then blocked with 5% nonfat milk in Tris-buffered saline-Tween (TBS-T) (20 mM Tris base (pH 7.6), 0.137 mM NaCl, and 0.1% Tween-20) for 1h at RT. Membranes were incubated with mouse monoclonal anti-hsp70 antibody (N27, a gift from Dr. W. J. Welch, University of Califonia, San Francisco, CA) for 1h at RT. After washing in TBS-T three times, the blot was incubated with peroxidase-conjugated secondary antibody for 1h at RT and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham).

Results

Temperature profile of heat shock response

In order to label the proteins responding heat shock with [^{35}S]-methionine, the hepatocytes were incubated at 21, 24, 27, 30, 32, and 35℃ for 2h, and labeled proteins were analyzed by SDS-PAGE and fluorography. Exposure of the cells to elevated temperatures caused the induction of hsp90, hsp70, hsp40, hsp30, and hsp27 synthesis (Fig. 1A). The synthesis of hsp70 were prominently enhanced by elevated temperatures. The enhanced synthesis of hsp70 was first observed at 21℃, its synthesis was continuously increased and was maximally induced at 32 or 35℃. Hsp90, hsp40, and hsp27 synthesis were increased at a much lower level compared to hsp70 and hsp30. For example, the levels of hsp90, hsp70, hsp40, hsp30, and hsp27 in heat-shocked hepatocytes at 32℃ increased approximately 4, 7, 3, 5, 2 fold, respectively, compared to control levels of each hsp (Fig. 1B). The synthesized protein profile showed that hsp70 and hsp30 were the major polypeptides induced in response to heat shock and hsp90, hsp40, and hsp27 were the minor polypeptides. The relative protein levels of hsp70 synthesized in cells exposed to various temperatures were examined by Western blot analysis with N27 antibody raised against human hsp70 (Fig. 2B). All gel lanes were loaded with an equal amount of total proteins (Fig. 2A). The levels of total hsp70 were increased at 30℃ and finally maximized at 35℃.

Effect of actinomycin D on hsp70 synthesis

In order to examine whether the synthesis of hsp 70 gene by heat shock is regulated at the transcriptional level, we carried out the protein labeling experiment and Northern blot analysis in cells treated with the transcriptional inhibitor actinomycin D prior to heat shock (Fig. 3). In protein labeling experiment, pretreatment of the actinomycin D completely inhibited the induction of hsp70 synthesis (Fig. 3A). Total RNA isolated from hepatocytes incubated in the absence or presence of actinomycin D was hybridized with a highly conserved sequence of flounder *hsp70*-related cDNA. *Hsp70* transcripts were also completely inhibited by pretreatment with actinomycin D in heat-shocked cells (Fig. 3B). These results suggest that expression of hsp70 gene is regulated at the transcriptional level.

Effect of variable duration of heat shock on hsps synthesis

We examined the synthesis of hsps in cells exposed to prolonged heat shock at various temperatures. The patterns of hsps synthesis were different from temperature and incubation time. When the cells was incubated at 30℃, hsp90, hsp70, hsp40, and hsp30 synthesis were induced. The most prominently enhanced synthesis of hsp70 by heat shock was detected within 2h and then maximized at 4h, but reduced to near control levels by 24h (Fig. 4A). The levels of hsp70 quantified by densitometric scanning increased approximately 3 fold at 2h, 9 fold at 4h, and 2 fold at 24h (Fig. 4C). A similar transient increase was also observed in hsp90, hsp40, and hsp30 synthesis. For example, the levels of hsp30 increased approximately 3 fold at 4h and gradually decreased to control levels by 10h (Fig. 4C). The levels of hsp27 was similar to those of control in all heat-shocked hepatocytes. This transient increase of hsps synthesis was also observed in cells exposed to prolonged heat shock at 24℃ or 27℃ (data not shown). Therefore, hsp90, hsp70, hsp40, and hsp30 synthesis were induced in a transient fashion at 30℃. However, when the incubation temperature of cells was maintained at 32℃, hsp70 and hsp30 synthesis were induced as a major band and synthesized continuously up to 24 h (Fig. 4B). As shown in Fig. 4D, the levels of hsp 70 and hsp30 at 24h increased approximately 12 and 25 fold, respectively, compared to control levels. Hsp90 and hsp40 synthesis were induced as a minor band up to 12h. The continuous synthesis of hsp70 and hsp30 observed for 24h at 32℃ is obviously contrast with the transient induction of

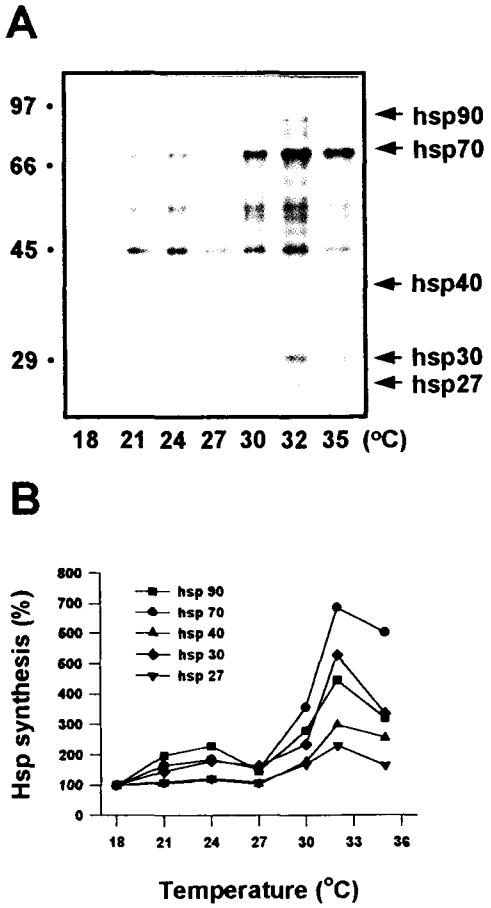

Fig. 1. Induction of hsps synthesis by heat shock in flounder hepatocytes. In panel A, the cells were grown at 18℃ prior to being shifted to higher temperatures. Cells were labeled with [^{35}S]-methionine at 18, 21, 24, 27, 30, 32, and 35℃ for 2h. The labeled proteins were analyzed by SDS-PAGE and fluorography. In panel B, levels of each hsp shown in the fluorography in panel A were quantifiedby densitometric scanning. For each sample equivalents amounts of acid-insoluble radioactivity were loaded in every case. Molecular weight markers (kD) were indicated on the left of the panel A and the positions of the hsps were indicated on the right of the panel A.

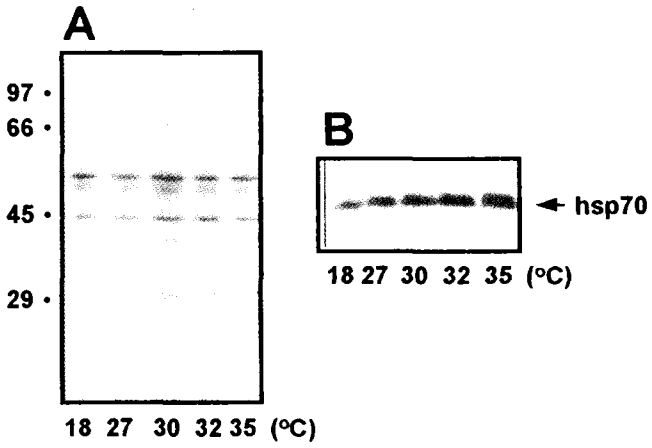

Fig. 2. Changes in hsp70 levels at elevated temperatures. Hepatocytes were grown at 18 ℃ prior to being shifted to higher temperatures. The cells were incubated at 18, 27, 30, 32, and 35℃ for 2h. The polypeptides were obtained from cells and analyzed by SDS-PAGE. The resulting gels were either Coomassie-stained (panel A) or Western blotted with anti-hsp70 antibody (panel B). Molecular weight markers (kD) were indicated on the left of the panel A and the position of hsp70 was indicated on the right of the panel B.

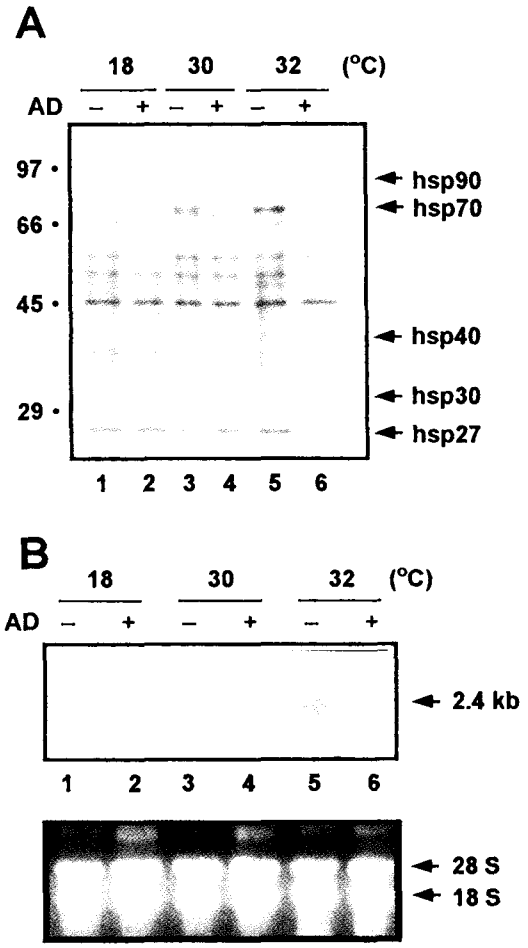

Fig. 3. Effect of actinomycin D on the hsp70 expression. In panel A, hepatocytes were incubated in the absence (lane 1, 3, and 5) or presence (lane 2, 4, and 6) of actinomycin D (AD, 20 μg/mℓ) for 30 min at 18℃ and then labeled with [^{35}S]-methionine at 18 (lane 1 and 2), 30 (lane 3 and 4), and 32℃ (lane 5 and 6) for 2h. The labeled proteins were analyzed by SDS-PAGE and fluorography. In panel B, RNA (25 μg) isolated from hepatocytes as described in panel A was eletrophoresed in an agarose gel, transferred to nylon membrane, and hybridized with a highly conserved sequence for flounder *hsp70*-related gene Arrow indicates a 2.4 kb corresponding to hsp70 transcripts. Ribosomal RNA (28S and 18S) as a control for equivalent loading and RNA integrity are shown at the bottom of the panel B.

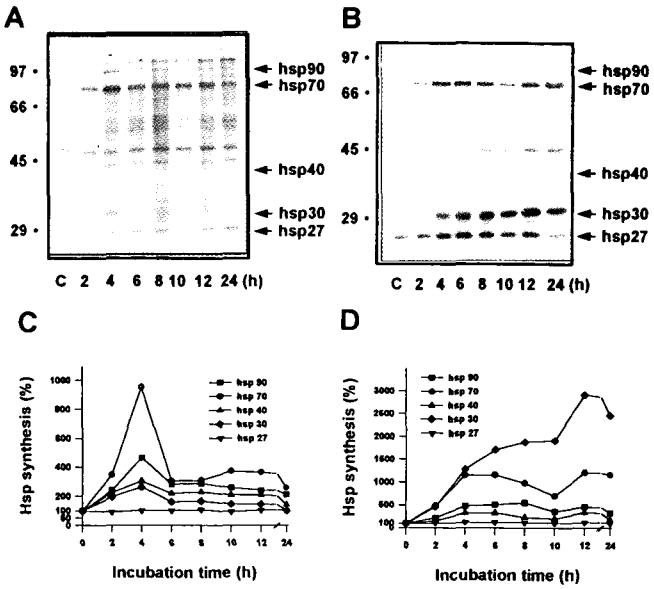

Fig. 4. Time course comparison of hsps synthesis in hepatocytes heat-shock treated at either 30 or 32℃. Hepatocytes grown at 18℃ were heat-shock treated at either 30 (panel A) or 32℃ (panel B) for 2, 4, 6, 8, 10, 12, and 24h and labeled with [^{35}S]-methionine for the last 2h. Control cultures (C) were labeled with [^{35}S]-methionine at 18℃ for 2h. The labeled proteins were analyzed by SDS-PAGE and fluorography. Panel C and D represent the densitometric scanning of hsps levels shown in the fluorography in panel A and B, respectively. Molecular weight markers (kD) were indicated on the left of panel A and B and the positions of the hsps were indicated on the right of panel A and B.

these hsps at 30℃. When cells were incubated at 35 ℃ for various periods of time, an enhanced synthesis of hsps were almost similar to that of hsps in hepatocytes exposed to prolonged heat shock of 32℃ (Fig. 5A). The levels of hsp90, hsp70, hsp40, hsp30, and hsp27 at 4h increased approximately 3, 12, 3, 10, 5 fold, respectively, compared to control levels of each hsp (Fig. 5B). Hsp70 and hsp30 only were synthesized as duration was prolonged (from 8h to 12h) and synthesis of most other proteins synthesized at 18℃ had ceased completely, which suggests that cell death happened in heat-shocked cells at 35℃ for 8h-12h. The most pronounced difference in the heat shock response between 32℃ and 35℃ was the induction of hsp27 synthesis at 35 ℃. Hsp27 synthesis was greatly increased by 2h and maximized by 4h. Hsp27 was synthesized continuously as a major band up to 6h. In order to examine whether hsp27 synthesis was induced at a higher temperature, cells were incubated at 37℃. Hsp27 synthesis was greatly increased at 2h and

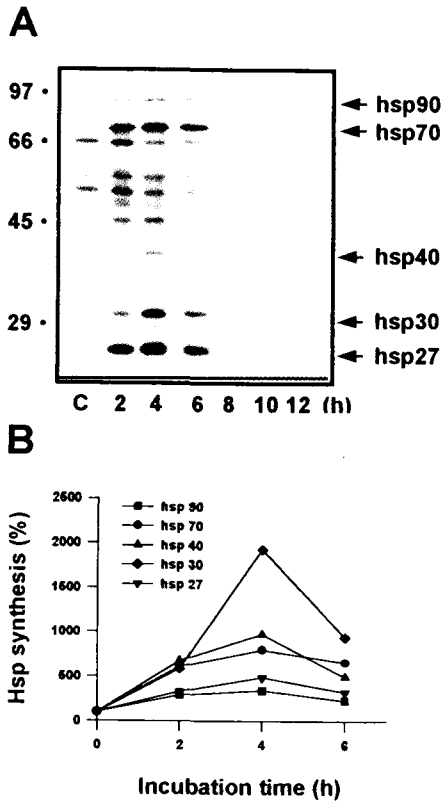

Fig. 5. Time course of hsps synthesis during incubation at 35℃. In panel A, hepatocytes grown at 18℃ were heat-shock treated at 35 ℃ for 2, 4, 6, 8, 10, 12, and 24h and labeled with [^{35}S]-methionine for the last 2h. Control cultures (C) were labeled with [^{35}S]-methionine at 18℃ for 2h. The labeled proteins were analyzed by SDS-PAGE and fluorography. In panel B, levels of each hsp shown in the fluorography in panel A were quantified by densitometric scanning. Molecular weight markers (kD) were indicated on the left of panel A and the positions of the hsps were indicated on the right of panel A.

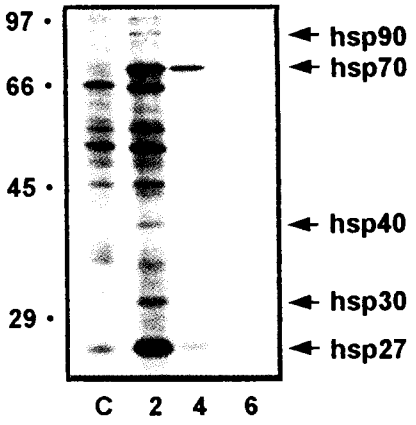

Fig. 6. Induction of hsp27 synthesis. Hepatocytes grown at 18℃ were heat-shock treated at 3 7℃ for 2h, 4h, and 6h and labeled with [^{35}S]-methionine for the last 2h. Control cultures (C) were labeled with [^{35}S]-methionine at 18℃ for 2h. The labeled proteins were analyzed by SDS-PAGE and fluorography. Molecular weight markers (kD) were indicated on the left of panel and the positions of the hsps were indicated on the right of panel.

its level quantified by densitometric scanning increased approximately 6 fold at 2h (Fig. 6). Hsp 90, hsp70, hsp40, and hsp30 synthesis were also induced at 2h. The decrease of cellular proteins synthesis at 4h means that cell death is happening.

Discussion

The heat shock response of flounder hepatocytes was investigated in this study. Five polypeptides of hsp90, hsp70, hsp40, hsp30, and hsp27 were induced at the elevated temperatures (Fig. 1). Hsp70 and hsp30 syntheses were induced as major polypeptides and hsp90 and hsp40 syntheses were also induced as minor polypeptides. Hsp27 was constitutively synthesized at normal temperature (18℃), but increased at a more severe temperature only (e.g., from 35℃ to 37℃). In flounder cells, the general trends of heat shock response are similar with goldfish caudal fin cell lines (RBCF-1, GTF e-3, GTF e-2) (Mitani et al, 1989; Sato et al, 1990) in sharing with hsp90, hsp70, and hsp30, and is common with rainbow trout white blood cells (Bols et al, 1992) in sharing with hsp90, hsp70, hsp40, and hsp27. The expression of hsp gene is mostly regulated at the transcriptional level (Gedamu et al, 1983; Kothary et al, 1984; Misra et al, 1989) although the stability or translational efficiency of hsp mRNAs was demonstrated to be involved in the expression of hsps (Lindquist, 1980a). Protein labeling experiment and Northern blot analysis showed that treatment of flounder cells with actinomycin D inhibited the induction of hsp70 synthesis by heat shock (Fig. 3 B). This suggests that expression of hsp70 gene in flounder cells is regulated at the transcriptional level. Elevation of flounder cells from 18 to 30, 32 or 35℃ for various periods of time increased the synthesis of a set of hsps. Hsp90, hsp70, hsp40, and hsp30 were transiently expressed at 30℃

(attenuation), but these hsps were continuously synthesized at 32℃ or 35℃. These results suggest that the differential pattern of hsps synthesis in flounder cells appears to be dependent upon the temperature used. The most noticeable example of this phenomenon was found with hsp70 and hsp30. At 30℃, the enhanced synthesis of hsp70 and hsp30 was maximized at 4h, but reduced to near normal level by 24h (Fig. 4A). However, hsp70 and hsp30 were continuously synthesized at maximal level even after 4h of heat shock at 32℃ (Fig. 4B). A temperature-dependent temporal pattern of hsp70 synthesis has been reported in *Drosophila* and Chinook salmon embryo cell lines (CHSE-214) (Lindquist, 1980b; Gedamu et al, 1983). At 24℃, maximal synthesis of CHSE-214 hsp70 occurred at 2h with recovery to near normal levels by 10h. However, hsp70 is continuously synthesized at maximal level even after 10-24h at 28℃. 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, 19 91). This attenuation phenomenon has been proposed to be mediated by heat shock transcriptional factor (Green et al, 1995). In flounder cells experiencing prolonged heat shock at 30℃, attenuation in the expression of hsps occurred. If this model is correct, the continued synthesis of hsps in flounder cells experiencing prolonged heat shock at 35℃ may be attribute to accumulation of the higher levels of heat-induced misfold polypeptides. The induction of hsp27 synthesis at 35℃ or 37℃ (Fig. 5 and 6) is similar to that of hsp 27 synthesis in RTG-2 cell lines exposed to 28℃ or 30℃ (Bols et al, 1992). In RTG-2 cell lines, hsp27 have been suggested to participate in cytoskeletal thermostabilization (Bols et al, 1992). The findings presented in this paper indicate that the optimal induction temperature of each hsp expression is clearly different, and that each hsp may be adapted differently to the protective mechanisms for heat stress within cells. Furthermore, an interesting observation in this study is that hsp30 synthesis was highly induced at high temperatures. This result suggests that this hsp may be involved in protection cells from injury at high temperatures. Our efforts are currently devoted to the isolation of this hsp and the investigation of regulatory mechanisms of hsp70 expression during the prolonged heat shock.

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