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Expression of a Gene Encoding Heat Shock Protein 70-Related Protein from Olive Flounder, *Paralichthys olivaceus*

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We have shown previously that the sequence of olive flounder (*Paralichthys olivaceus*) *hsp70*-related cDNA has a high similarity with those of cognate *hsc70* of other species (Kim et al., 1999; J. Aquaculture, 12 : 91-100). In order to investigate whether this gene encodes the cognate hsc70, we examined the expression of this gene in normal and heat-shocked conditions. By *in vitro* translation, this gene encoded a 70 kD protein which was constitutively expressed and was not induced by heat shock. This translated protein was recognized by anti-hsp/hsc70 antibody. Tests of heat-inducibility showed that this gene was constitutively expressed in normal conditions and its expression was not increased after heat shock. The expression levels of this gene were high in stomach, gill, intestine, kidney and brain, moderate in liver, and comparatively low in ovary and heart. Furthermore, Northern blot analysis of transcript expression showed that the corresponding mRNA were detected throughout embryonic development in the absence of any heat shock. These results provided evidence that olive flounder *hsp70*-related cDNA encoded to a cognate member of hsp70 family, hsc70.

Key words : Olive flounder, *Paralichthys olivaceus*, *Hsp70*-related cDNA, *In vitro* translation, Northern blot analysis, Embryonic development

Introduction

Following heat shock or a variety of other stresses, all cells and organisms respond by synthesizing a group of proteins called heat shock proteins (hsps) (Misra et al., 1989; Welch, 1990). These proteins are among the most highly conserved throughout evolution. Although a limited number of species have been examined, the fish hsps can be grouped into six families based upon their molecular masses: 100 kD (hsp100), 84-95 kD (hsp90), 65-76 kD (hsp70), 59-62 kD (hsp60), 39-42 kD (hsp40), and 27-30 kD (hsp27) (Mooser et al., 1986; Chen et al., 1988; Bols et al., 1992). Among the different classes of hsps, the largest group of hsps is the hsp70 family, which is comprised of both heat (stress) inducible and constitutively expressed (cognate) members (Lindquist and Craig, 1988). A hsp70 expressed in unstressed cells under physiological conditions is called hsc70 (heat shock cognate 70) (Craig et al., 1993). Such constitutive expression of *hsc70* gene has been reported in a wide variety of nonstressed eukaryotic cells from *Drosophila*, amphibian, fish, mouse, and human (Bensaude et al., 1983; Craig et al., 1983; Bienz 1984; Dworniczak and Mirault, 1987; Arai et al., 1995).

The role of hsp/hsc70 protein during the heat shock response has been extensively studied over a number of years (Georgopoulos and Welch 1993; Hartl, et al., 1994). Indeed, hsc70 is essential for cell viability under normal growth conditions. It functions as a molecular chaperone which regulates protein folding, transport, and assembly in both eukaryotic and prokaryotic systems (Ellis and Van der Vies, 1991; Gething and Sambrook, 1992). In addition, it is known that hsc/hsp70 facilitates a post-translational protein translocation across the membrane of endoplasmic reticulum or mitochondria (Chirico et al., 1988; Deshaies et al., 1988). Many investigators have suggested that besides their function as molecular chaperone, hsc70 might be involved in the embryonic development. In *Drosophila*, Xenopus, and zebrafish, for example, hsc70 were found to be differentially expressed during embryogenesis (Craig et al., 1983; Bienz, 1984; Santacruz et al., 1997).

Recently, we have isolated and characterized a olive flounder *hsp70*-related cDNA, and have showed that the nucleotide and amino acid sequence of this gene have a high similarity with those of cognate *hsc70* of other species (Kim et al., 1999). Whether a heat shock gene is heat-inducible (*hsp* gene) or cognate (*hsc* gene) depends on the regulation of the gene in normal conditions: a heat-inducible gene is efficiently expressed only after heat induction, whereas a cognate gene has a high basal level of expression and is not or is weakly heat-inducible (Mckay, 1993). In the present study, in order to investigate whether olive flounder *hsp70*-related cDNA encodes the cognate hsc70, we examined the expression of this gene in normal and heat-shocked conditions using this gene as probe.

Materials and Methods

Fish and embryo culture

Olive flounders were obtained from Koje Hatchery of National Fisheries Research and Development Institute and maintained in 6 tons flow-through tank at 18±1℃ under a natural photoperiod. Embryos were obtained from natural matings and reared in filtered seawater. Embryogenesis was processed synchronously among batches of embryos. Larvae hatched about 70-72 hr after fertilization. Samples at appropriate stages were collected and total RNA was extracted for Northern blot analysis. Oocytes were obtained from the matured female olive flounder.

Preparation of hepatocytes, cell culture, and heat shock

Hepatocytes were isolated by a modification of the two-step hepatic perfusion procedure described by Seglen (1977). The cells were resuspended in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and seeded onto tissue culture flasks. For metabolic labeling, cells grown routinely at 18±0.1℃ in regulated air-incubators for 24h were incubated at 18 and 32℃ for 2h. For tests of heat inducibility, cells were incubated at 30 or 32℃ for 2h. For prolonged heat shock, cells were incubated at 30℃ for varying periods of times as indicated in the legend of figure 3.

Metabolic labeling and sodium dodecyl sulfate-polyacryamide gel electrophresis

For metabolic labeling, cells incubated at 18 and 32℃ for 2h were washed with methionine-free Dulbecco's modified Eagles medium (DMEM)

and cellular proteins were labeled with [^{35}S]-methionine (62 μCi/ml) for 2h. Then cells were washed three times with phosphate-buffered saline 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 (LSC). Equivalent amounts of radioactivity were loaded for each sample. SDS-PAGE was carried out on 12.5% polyacrylamide gel as described by Laemmli (1970). After electrophoresis, one part of gel was 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. Other part of gel was transferred to membranes for Western blot analysis.

In vitro translation and Western blot analysis

Denatured olive flounder *hsp70*-related cDNA (10μg) was immobilized on a nitrocellulose filter and hybridized with 30μg of poly(A^+)-selected RNA isolated from normal and heat-shocked hepatocytes in 50% formamide, 0.9 M NaCl, 0.2% SDS, 1 mM EDTA, and 20 mM PIPES (pH 6.4) for 6h at 37℃. Filters were washed 10 times in 1×SSC, 0.5% SDS for 5 min at 60℃. Three final washes were done in the same buffer without SDS. Hybridized RNA was eluted thermally and ethanol precipitated. Hybrid-selected RNA was translated *in vitro* by using rabbit reticulocyte lysate (Rabbit Reticulocyte Lysate Systems, Promega) in the presence [^{35}S]-methionine. Radiolabeled products were analysed by SDS-PAGE through 12.5% polyacrylamide gel and the dry gel was fluorography as described above. Western blot analysis was performed as described by Towbin et al. (1979). The electrophoresed gels were transferred to Hybond-N membrane (Amersham) 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 room temperature (RT). Membranes were incubated with mouse monoclonal anti-hsp/hsc70 antibody (N27, a gift from Dr. W. J. Welch, University of Califonia, San Francisco, CA), which recognizes both inducible hsp70 and cognate hsc70, for 1h at RT. The N27 epitope is in the region of amino acid 503-640 of human hsp70. After washing in TBS-T three times, the blots were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) for 1h at RT. The blots were developed in alkaline phosphate buffer (100 mM NaCl, 5mM $MgCl_2$, 100 mM Tris base (pH 9.5)) containing nitro blue tetrazolium and bromo-chloro-indolyl phosphate.

RNA preparation

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

Preparation of probes

In the *hsp70*-ralated gene family, the highest conserved part of the sequence recognizes the both *hsp70* and *hsc70* gene. However, as the least conserved part of the sequence was likely to be gene-specific (Chappell, et al., 1987), the very 3' end of olive flounder *hsp70*-related gene recognizes this *hsp70*-related gene only. Therefore, in order to investigate the expression of this gene in normal and heat-shocked hepatocytes, we amplified both the highest conserved part (corresponds to 1183-1542 of the flounder *hsp70*-

related cDNA sequence) and flounder *hsp70*-related cDNA-specific part (correspondes to 1906-2250 of a olive flounder flounder *hsp70*-related cDNA sequence). Oligonucleotides designed according to a olive flounder *hsp70*-related cDNA (accession number AF053059) (Kim et al., 1999) were used for RT-PCR amplification of cDNA prepared from total RNA of the hepatocytes by oligo (dT)-primed reverse transcription according to the manufacturer's instructions (GIBCO/BRL). RT-PCR for the highest conserved sequence was performed using the forward (5'-GATGAAGCTGTGGCCTACGGAG-3') and the reverse (5'-GATGCCGTTGGCATC-AATATC-3') primers. The PCR cycle consisted of 1 min denaturation at 94℃, 1 min annealing at 55℃, and 1 min extension at 72℃ for 35 cycles. RT-PCR for the specific sequence was performed under the same conditions as described above using the forward (5'-ATCACTA-AGCTGTACCAGAGTG-3) and the reverse (5'-GGCCACGCGTCGACTAGTAC $(T)_{17}$-3') primers. Expected PCR products were cloned into the TA cloning vector pCR2.1 (Invitrogen), sequenced, and used as probes for Northern blot analysis.

Northern blot analysis

Total RNA (25μg) from hepatocytes, various tissues, oocytes and embryos from olive flounder 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 probes were 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 1 mM EDTA) at 42℃ for 14hr. Membranes were washed in 2 X SSC-0.1% SDS at 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℃.

Results and Discussion

In vitro translation of olive flounder hsp70-related RNA

In order to demonstrate that the *hsp70*-related cDNA clones we isolated previously (Kim et al., 1999) encode a protein of the hsp70 family, we selected a olive flounder *hsp70*-related mRNA by hybridization of both hepatocytes and heat-shocked hepatocytes poly(A^+) RNA to olive flounder *hsp70*-related cDNA clones immobilized on nitrocellulose. The [^{35}S]-methionine-labeled translation products of this selected RNA were analyzed by SDS-PAGE and Western blot analysis with the N27 (Fig. 1). This N27 antibody, which was raised against a human hsp70, recognizes both inducible hsp72 and cognate hsc73 protein. Two isoforms of flounder hsp70, hsp70 and hsc70, were found (Fig 1A, lane 2) and recognized by the N27 antibody. (Fig. 1B, lane 2). The larger isoform was constitutively expressed and its synthesis was not changed much by heat shock. The expression of smaller isoform was greatly increased by heat shock. The molecular mass of the translated protein in hepatocytes and heat-shocked hepatocytes RNA was found to be approximately 70 kD. And this protein was migrated at a higher molecular mass than the band of the inducible hsp70 (Fig 1A. lane 3 and 4). The other minor translation products were also recovered. However, only the major product of 70 kD was specifically recognized by the N27 antibody specific to the hsp/hsc70 protein. This result demonstrates that

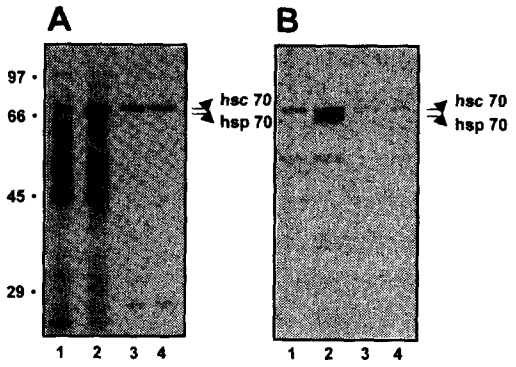

Fig. 1. *In vitro* translation of olive flounder *hsp70*-related RNA. In panel A. lane 1 and 2 represent [^{35}S]-methionine-labeled proteins isolated from normal (18℃) and heat-shocked hepatocytes (32 ℃), respectively. Lane 3 and 4 represent [^{35}S]-methionine-labeled *in vitro* translation products of RNA from normal and heat-shocked hepatocytes, respectively, selected by hybrid arrest a with flounder *hsp70*-related cDNA clones. In panel B, lane 1 and 2 represent Western blot of [^{35}S]-methionine-labeled proteins isolated from normal and heat-shocked hepatocytes, respectively, with N27 antibody. Lane 3 and 4 represent Western blot of [^{35}S]-methionine-labeled *in vitro* translation products from normal and heat-shocked hepatocytes, respectively, with N27 antibody. Both forms of hsp70 and hsc70 are clearly distinguishable by their molecular mass and are indicated by arrow on the right of each panel. Molecular weight markers(KD) are indicated on the left of the panel A.

the cDNA clones encode a constitutively expressed hsp70-related protein which is not induced by heat shock.

Heat-inducibility of olive flounder hsp70-related transcripts

To examine precisely whether transcripts related to a olive flounder *hsp70*-related cDNA were induced by heat shock, we carried out experiments for heat-inducibility in normal (18 ℃) and heat- shocked hepatocytes (30 or 32℃) using Northern blot analysis. While the flounder *hsp70*-related gene-specific probe from the very 3'-end of the cDNA detected a single transcript of about 2.4 kb whose intensity did not increase after heat shock (Fig. 2A), an increase in the amount of transcript was observed with the probe from the highest conserved portion (Fig. 2B), suggesting that the induction of the transcripts was due to that of the inducible *hsp70* transcripts. This transcript was also constitutively expressed in nonstressed hepatocytes. These results are consistent with previous data of Zafarullah et al (1992), who found constitutive *hsc70* expression in CHSE cells using rainbow trout *hsc70* gene as probe. The expression of flounder *hsp70*-related transcripts during prolonged heat shock at 30℃ was also examined. In hepatocytes using specific probe for flounder *hsp70*-related gene, *hsp70*-related transcripts

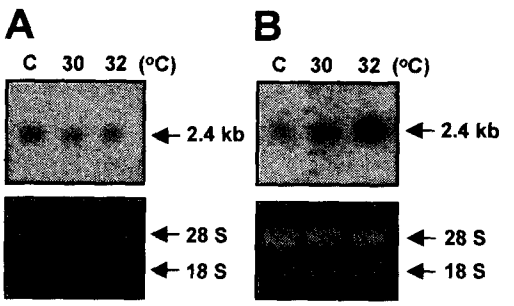

Fig. 2. Heat-inducibility of olive flounder *hsp70*-related gene. RNA (25μg) isolated from normal (18℃, C) and heat-shocked (30 and 32℃) hepatocytes was eletrophoresed in an agarose gel, transferred to nylon membrane, and hybridized with specific sequence (nucleotides 1906-2250) for *hsp70*-related gene (panel A) and the highest conserved sequence of the gene (nucleotides 1183-1542) (panel B). Arrow indicates a 2.4 kb corresponding to *hsp70*-related transcripts. Ribosomal RNA (28S and 18S) as a control for equivalent loading and RNA integrity are shown at the bottom of each panel.

were also constitutively expressed in normal condition and were not changed during prolonged heat shock (Fig. 3A). However, in hepatocytes using the highest conserved part of the sequence as probe, the transcripts were increased by 2h, maximally by 4h, and gradually attenuated during prolonged heat shock (Fig. 3B), suggesting that the induction of transcripts was also due to that the inducible *hsp70* transcripts.

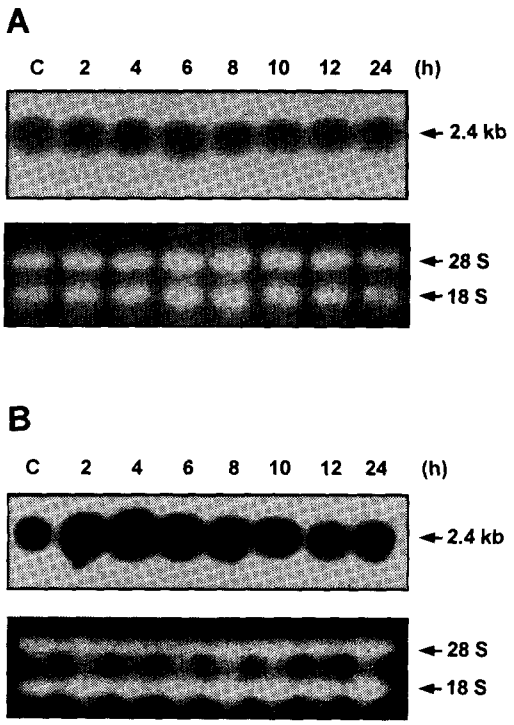

Fig. 3. Expression of olive flounder *hsp70*-related transcripts during prolonged heat shock. RNA (25μg) isolated from normal (18℃, C) and heat-shocked hepatocytes (30℃) for the times indicated was eletrophoresed in an agarose gel, transferred to nylon membrane, and hybridized with specific sequence (nucleotides 1906-2250) for *hsp70*-related gene (panel A) and the highest conserved sequence of the gene (nucleotides 1183-1542) (panel B). Arrow indicates a 2.4 kb corresponding to hsp70-related transcripts. Ribosomal RNA (28S and 18S) as a control for equivalent loading and RNA integrity are shown at the bottom of each panel.

Constitutive expression of olive flounder hsp70-related transcripts in various tissues

To analyze the constitutive expression levels of the *hsp70*-related transcripts in different flounder tissues, total RNA isolated from various tissues was hybridized with specific probe for flounder *hsp70*-related gene. A single transcript of 2.4 kb was detected and found to be constitutively expressed in all tissues examined (Fig. 4). The levels of this transcript were high in stomach, gill, intestine, kidney, and brain, moderate in liver, and comparatively low in ovary and heart. This result showed that flounder *hsp70*-related gene is constitutively expressed under normal conditions.

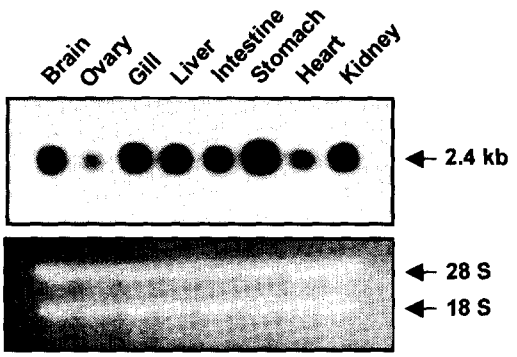

Fig. 4. Expression of olive flounder *hsp70*-related transcripts in various tissues. RNA (25μg) isolated from brain, ovary, gill, liver, intestine, stomach, heart, and kidney tissues was eletrophoresed in an agarose gel, transferred to nylon membrane, and hybridized with specific probe for *hsp70*-related gene (nucleotides 1906-2250). Arrow indicates a 2.4 kb corresponding to *hsp70*-related transcripts. Ribosomal RNA (28S and 18S) as a control for equivalent loading and RNA integrity are shown at the bottom.

Differential expression of olive floundr hsp70-related transcripts during embryonic development

To investigate the temporal expression pattern of flounder *hsp70*-related transcripts in embryonic development, RNA isolated from oocytes and embryos at different stages of the development was hybridized with specific probe for flounder *hsp70*-related gene. A single transcript of 2.4 kb was detected at oocytes and at different stages of embryos (from the 2-4 cells to the 30-35 myotomes) (Fig. 5). *Hsp70*-related transcripts were shown to be expressed in normal conditions throughout embryonic development. This result is are consistent with that obtained in other species, since it was reported that in zebrafish, transcripts of the cognate *hsc70* gene were found throughout embryonic development (Santacruz et al., 1997). Furthermore, in *Drosophila*, an *hsc70.4* gene was also shown to be expressed under normal conditions in embryos (Perkins et al., 1990). The expression of h*sp70*-related transcripts was rapidly increased from the stage of gastrula and reached a maximum level at the 12-14 myotomes stage, and then maintained the maximal level until 30-35 myotomes stage.

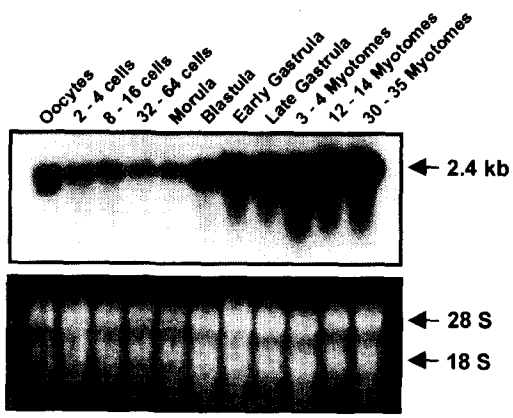

Fig. 5. Expression of olive flounder *hsp70*-related transcripts during embryogenesis. RNA (25μg) isolated from ovaries, 2-4 cells, 8-16 cells, 32-64 cells, morula, blastula, early gastrula, late gastrula, 3-4 myotomes, 12-14 myotomes, and 30-35 myotomes stage of embryos was eletrophoresed in an agarose gel, transferred to nylon membrane, and hybridized with specific probe for *hsp70*-related gene (nucleotides 1906-2250). Arrow indicates a 2.4 kb corresponding to *hsp70*-related transcripts. Ribosomal RNA (28S and 18S) as a control for equivalent loading and RNA integrity are shown at the bottom.

In zebrafish, the midblastula transition defined by Kane and Kimmel (1993) take place at the 512-cell stage of development. The onset of the midblastula transition is marked by cell cycle lengthening. Furthermore, as interphase lenghens, transcription increases over the background level. Although the molecular mechanisms about developmental process of flounder are rarely studied, increase of flounder *hsp70*-related transcripts from the stage of gastrula may due to that of transcription of flounder *hsp70*-related gene followed the onset of the midblastula transition as in zebrafish. The *hsp70*-related transcripts detected during cleavage stage in flounder may be of maternal origin, as in zebrafish and in *Xenopus*, since no zygotic transcription occurs before the midblastula transition (Ali et al., 1996; Santacruz et al., 1997). Such a hypothesis is consistent with the fact that *hsp70*-related gene is strongly expressed in mature oocytes. The maternal *hsp70*-related transcripts were present, but it did not increase during the cleavage stages. The levels of maternal *hsc70* transcript were maintained until morula stage.

Results from *in vitro* translation, heat-inducibility, expression in various tissues, and expression during embryogenesis for flounder *hsp70*-related gene provided evidence that flounder *hsp70*-related gene encodes to a cognate

member of hsp70 family, hsc70. In particular, differential expression during embryogenesis provides important research information which will be useful for studying the role of this protein during embryogenesis in flounder

Acknowledgments

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