Cloning and characterization of cDNAs for 70-kDa heat-shock proteins (Hsp70) from two fish species of the genus Oryzias

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ABSTRACT

cDNA corresponding to two hsp70-related genes (OLHSC70 and CEHSC70) were isolated from two lines of cultured fish cells derived from the genus Oryzias. OLHSC70 was 2,261 bp in length and encoded a protein of 686 amino acids with a predicted molecular mass of 76,120 daltons. CEHSC70 was 2,114 bp in length and it lacked the 5' region found in OLHSC70. Two-dimensional electrophoresis revealed that Oryzias latipes has at least three heat-inducible proteins with molecular masses of about 70,000 daltons. One of these proteins (Hsp70.1) was barely expressed under normal conditions but its high-level expression was induced by hypertermia. The other two proteins (Hsc70.1, and Hsc70.2) were constitutively expressed under normal conditions and only slightly enhanced levels were induced by hypertermia. Transfection with the cloned sequence, RNA dot-blot analysis and the two-dimensional electrophoresis of proteins showed that OLHSC70 encoded Hsc70.1.

1. INTRODUCTION

Heat shock proteins (HSPs) are synthesized in response to an increase in temperature above physiological levels, and also by other environmental stresses, such as chemicals, adenovirus infection and anoxia (Ashburner and Bonner, 1979; Schlesinger et al., 1982). The 70-kD heat-shock protein (HSP70) is the most abundant HSP and its structure is remarkably conserved among various organisms (Morimoto, 1990). There are several hsp70-related genes, some of which are induced by environmental stress and some of which are not (Morimoto, 1990). In some studies it has been demonstrated that these proteins induce the thermostolerance and are associated with the thermostensitivity of the host organisms (Subjec et al. 1982; Ohtsuka and Laszlo, 1992).

The medaka, Oryzias latipes is an eurythermic fish, which can survive in water at temperatures from 4°C in winter to 40°C, a temperature here is sometimes reached in summer. A related species, O. celebensis, is a tropical fish found in Sulawesi, Indonesia (Naruse et al., 1993). The patterns of synthesis of HSPs in

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isolated fins of *O. latipes* change with the temperature of acclimatization (Oda et al., 1991). Kubota et al. examined the thermosensitivity of embryos and found that those of *O. celebensis* were more resistant to heat treatment than those of *O. latipes* (unpublished data). In addition, two lines of cultured fish cells derived from these two species have different degrees of thermosensitivity and cultured cells derived from *O. celebensis* are more resistant to heat treatment than those from *O. latipes*. The patterns of expression of heat-shock proteins, in particular HSP70s, also differ between these two species (Arai et al., 1994). In an attempt to understand the mechanisms of thermosensitivity of these two species of fish, we isolated *hsp70*-related genes and characterized them by DNA hybridization studies and two-dimensional electrophoresis of encoded proteins.

2. MATERIALS AND METHODS

Cell culture

OL32 cells were derived from fins of *O. latipes* (Komura et al., 1988), while CE cells were derived from an embryo of *O. celebensis* (Kubota personal communication). Cells were cultured at 33°C in a HEPES-buffered (10 mM) L-15 medium supplemented with 15% fetal calf serum and 60μg/ml kanamycin.

Construction and screening of libraries

Cells were heat-shocked in prewarmed water baths (41°C for OL32, 42°C for CE) for 1 h, and then incubated at 33°C for 1 h. The cDNA libraries were constructed using a ZAP-cDNA Synthesis kit (Stratagene). Insert of isolated clones were excised and recirculized to pBluescript by the Zap system as described in the instruction manual. We used 8×10⁷ cells to construct a cDNA library. The temperatures of the heat-shock treatment were determined from the extent of accumulation of heat-inducible transcripts (see Results and Discussion).

To obtain a probe for screening, we used a part of a *hsp70*-related sequence that had been amplified by two series of polymerase chain reactions with reverse transcription (RT-PCR: Innis et al., 1990). cDNA was prepared from heat-shocked cells using a Micro-Fast Track mRNA isolation kit (Invitrogen) and a cDNA synthesis kit (Boehringer Mannheim) for preparation of a template for PCR. Heat-shock conditions were the same as described above. The primers for PCR were designed from a comparison of *hsp70*-related sequences from Xenopus (Bienz, 1984), chick (Morimoto et al., 1986), mouse (Giebel et al., 1988; Zakeri et al., 1988; Hunt and Calderwood, 1990) and human (Dworniczak and Mirault, 1987; Hunt and Morimoto, 1985). Three conserved regions were chosen initially as primer regions. The sequences of the primers were as follows: P1, GCCAACGACCAGGGCAACCGCACCA; P2, GAAAGGCAGTGTCCATGTC; and P4, CCATACAGGATTTATGCTCTTTGTT.

For the first PCR, the template was cDNA from OL32 and the primers were P1
and P2. The product formed a single band of 190 bp (190-bp PCR product), and the nucleotide sequence was determined. The P3 primer (CGATTTGTGATCGATTTGACAGT) was designed on the basis of the sequence of the product of PCR amplified from primers P1 and P2. Amplification by PCR was performed in a total 50 μl of 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 0.2 mM deoxynucleoside triphosphates, 10 μM each primer, 1 μg of template DNA and 1 unit of Taq DNA polymerase. The reaction was carried out by heating at 93°C for 45 sec, with annealing at 65°C for 45 sec and elongation at 72°C for 60 sec. This cycle was repeated 40 times (Innis et al., 1990). For the second PCR, the template was cDNA from CE cells and the primers were P3 and P4. A product of about 800 bp was obtained (800-bp PCR product). With this 800-bp PCR product as a probe, RNA dot-blot analysis was carried out. The probe hybridized with samples from non-heat-shocked and heat-shocked cells (data not shown). Then this probe was used as the probe for isolation of hsp70-like cDNA s from cDNA libraries.

Sequencing and analysis of sequences

Nucleotide sequences were determined with a Sequenase kit version 2 (United States Biochemical Corp.). A homology search was carried out with DNASIS (Hitachi Software Engineering Co. Ltd.). Alignments were performed using CLUSTAL V (Higgins and Sharp, 1988). MEGA (Molecular Evolutionary Genetic Analysis Version 1 : Kumar et al., 1993) was used for construction of a phylogenetic tree.

The nucleotide sequence of OLHSC70 reported in this paper will appears in DDBJ, EMBL, and Genbank Nucleotide Sequence Database with accession numbers of D13669.

RNA dot-blot analysis

Total RNA was transferred to a Biodyne nylon membrane (Pall) using a microscale sample filtration manifold device. Ten μg of RNA were blotted in each slot. DNA probes were labeled with [³²P]dCTP by the random primer method (Feinberg and Vogelstein, 1983). The membrane was prehybridized with hybridization buffer (5×SSPE, 5% Irish Cream Liqueur (Baileys), 10 μg/ml denatured calf thymus DNA, 5% SDS) at 65°C for 1 h. Then the membrane was allow to hybridize with a radioactive probes in hybridization buffer at 65°C overnight. After hybridization, the membrane was washed once with 5×SSC that contained 1% SDS at room temperature for one min, and twice with 2×SSC that contained 1% SDS at 65°C for 30 min. The signals were detected by autoradiography on X-Omat film (Kodak).

Transfection by electroporation

The insert from an isolated cDNA clone, OLHSC70, was ligated with the expres-
sion vector, pcDNAI (Invitrogen). The resultant plasmid, pcOLHSC70, was introduced by electroporation into OL32 cells by the method of Hayasaka et al. (1990).

**Two-dimensional electrophoresis of proteins**

To prepare the sample for two-dimensional electrophoresis, cells were suspended in sterilized distilled water at a concentration of $10^6$ cells/ml, and then they were frozen and thawed three times. The mixture was then centrifuged to remove cell debris. Two-dimensional electrophoresis was performed by the method of O'Farrell (1975) with slight modification (Wang et al., 1993). After electrophoresis, gels were stained with a silver-staining kit (Daiichi Pure Chemical).

3. RESULTS AND DISCUSSION

**Cloning of cDNAs for hsp70-like genes**

A hsp70-related sequence was amplified by RT-PCR from CE cells. Using this product as a probe, we screened cDNA libraries derived from OL32 cells of *O. latipes* and from CE cells of *O. celebensis*. Several positive clones were isolated and analyzed by digestion with restriction enzymes. We select one clone from the cDNA library of OL32 cells and one clone from the cDNA library of CE cells. DNA sequence analysis showed that the clone, CEHSC70, from the cDNA library of CE cells lacked the 5' region of the non-coding and coding regions, while clone, OLHSC70, from the cDNA library of OL32 cells contained the complete coding region. The nucleotide sequences of OLHSC70 and CEHSC70 were shown in Fig. 1. OLHSC70 had a 5' non-coding leader sequence of 121 nucleotides, a 3' non-coding region of 79 nucleotides, and it encoded a protein of 686 amino acids with a predicted molecular mass of 76,120 daltons. CEHSC70 was 2,114 nucleotide long and lacked the 5' region of OLHSC70. The two sequences were about 90% homologous. The extent of the nucleotide homology in the 3'-non-coding region was about 80% and several deletions or insertions were appeared when the two sequences were compared. We isolated two other hsp70-related cDNAs from the cDNA library of CE cells but the homology between OLHSC70 and these two cDNAs was lower than that between OLHSC70 and CEHSC70 (data not shown). Our results indicate that OLHSC70 and CEHSC70 were homologs.

**Characterization of the hsp70 genes**

To characterize our clones related to the hsp70 genes, we constructed a phylogenetic tree of the members of the hsp70 gene family from seven species of vertebrate. In addition, we examine the patterns of expression of hsp70-related genes using two probes ( probe A, the common coding region of the hsp70 family and probe B, specific for the 3' region of OLHSC70) and we analyzed the patterns
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of expression of HSP70 proteins in OL32 cells with or without transfection with pcOLHSC70 (OLHSC70 cDNA ligated to the eukaryotic expression vector pcDNA 1) by two-dimensional electrophoresis.

Figure 2 shows a phylogenetic tree constructed from protein sequences

1 G GAA TTC GCC AGC AGG TTT TTT TTT TTT ACT CTC CTC GTC CGG GTA AGC TCC GAG TAC CTC GTC 58
59 GCC CTC GCT GCT CTC CGG TTT CCA AAC ACA CAC TTT CCA CGG ACC ATC TAT TTT GGA 
60 # T T 
61 (A) 119 AGC ATG CCT ATG GCC AAG ATG GTT GAA ATC AGT CCA ATC GAC TAC TGC TGT GAT 
62 (B) 178 C T G
63 (C) 1 M W S K G P A V G I D L G T T Y S C V G 
64 19 A A A P1
65 179 GTG TTC CAG CAT GCC AAG GTT GAA ATC ATC GCC AAT GAT CAG GGT AAT AGC ACC ACA CCA 
66 238 20 V F Q H G K V E I I A N D Q G N R T T P C
67 39 5 C T C
68 239 ACT ATG TGC TTC ACC GAT ACA AGG AGG CCT ATC GGA GTT GCA GCC AAA ACG AGT 
69 298 40 S Y V A F T D T R L I G D A A K N Q V 
70 59 A A A C
71 299 GCA ATG AAC CCC ACC AAC ACC TTT GAT GCC AAG AGC ATC CTT GGG GCA AGA TTC GAT 
72 358 60 A M N P T N T V F D A K R L I G R R F D 
73 79 P3 G C P2 GC T G
74 359 GAT CAT GTC GAC TCT GAC AGC CAT GTC CCT ATT GTA ATC AAT GAC AAG ACC 
75 418 80 D H V Q S D M N/K D/R W F P F N/A V I N D N T/S 
76 99 T T A C A
77 419 CTT CAA ACC GTC CAG GTC GAC TAA GAA GGA ACA AAG TCC TAT CCT GAG GAT 
78 478 100 R P K V Q V E Y K G E T K S/P F Y P E E V/I
79 119 G T
80 479 TCT TCA AGT GTG TGG ACA AAA ATG AAG GAT GCA GCC TAC TCT GGA AAA ACG GTC 
81 538 120 S S M V L T K M R E I A E A Y L G K T V 
82 139 T T A C A
83 539 AAC AAT GCT GTT ATC ACA GTA CCA GCC TTC TAC TAT GAC TCA GAC TCA GGA ACC AAC 
84 598 140 N N A V I T Y V P A Y F N D S Q R Q A T K 
85 159 C A
86 599 GAT GCC GCC ACA ATC TCT GGT CTT AAT GGT CTC ATC ATC AAT GCA CAA ACT ACT GCT 
87 658 160 D A G T I S G I L N V L R I N E P T A A T 
88 179 C T A C T C T
89 659 GCA ATG GCC TAT GGG CTG GAC AAA AAG GTT GCC TCC CAG AAT GTT CCT ATC TTT GAT 
90 718 180 A I A Y G L D K K V G S E R N V L I F D 
91 199 G G C T T A C A
92 719 CTT GGT GGT GCC TAT TTT GAT GTC TCT ATC ATC ATT GAT GAT GAT ATT TTT GAC GTC 
93 778 200 L G G/G A G T/1 F D V S I L I T E D G I F E V 219 A A A G
94 779 AAG TCT ACT GCT GGA GAC ACT CAT CTT GGT GAT GAA GAT TTT GAC AAG GCC ATG AGT 
95 838 202 K S T A G D T H/N L G E/G D F D N R M V N 
96 239 T C N N N N N T
97 839 CAC TTC ATC GCA GAG TTT AAA GCC AAA AAG GAA TAC GAC ACA ATG ACT ACG 
98 998 240 H F I A E/X F/X K K K Y K X X X D I S D N K R A 
99 259 T A A A C G N
100 899 GTC CGG GCC CGC TTC CTC GCT GCT CCT GGT GAG AGC GAG ACA CGA ACA CTG TCC TCC ACC ACT CAA 
102 279 N N C C C C C
103 959 GCA ATT ATT CCT GAT TCC TAC GAG GGA GTC GAT TAT ACG ATC ATC ACC AGG 
104 1018 280 A S E I E D S/P L Y E G V/X D/X F Y T S I T R T N N N C C A A
105 299 1019 GCA GGT TTT GAT GAC GCT CAA GCC ATC CCT GCA GCC ACC ATC GAT CTT GCG GAG 
106 1078 300 A R F E E L N A D L P/X R/X G T L D P V E K E G T C C C
107 1079 TCA CCT GAT GGT GAG ATG AAA GTA TTT GTT ATT CAT GAT GAT TTA GTT GGC GGT 
109 339 T A M N N N N C C N N G N 
110 1139 TCC ACT GCC ATC CGG AAG AGT ACA AAA CTA CCA GAC TCT TTT ATT GGA AAA GAG CTC 
111 1198 340 S T R I P K I/X Q/X K L L Q/X D A P/X F N G K E/X L 
112 359
Fig. 1. Nucleotide sequences of CEHSC70 (A) and OLHSC70 (B) cDNAs. The predicted amino acid sequence (C) is indicated below the DNA sequence. Only differences between CEHSC70 and OLHSC70 are indicated. P1, P2, P3 and P4 show the primer-annealing sites for each primer on OLHSC70. An asterisk indicates the first nucleotide of CEHSC70. Termination codons are shown in italics. Probe B is shown in a double-underline.
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Fig. 2. A phylogenetic tree of members of HSP70 family from 7 vertebrates as deduced by the neighbor-joining method (Saitou and Nei, 1987). Constitutively expressed HSP70 cognate genes are as follows; MUSHSPCA (M1914, Mus musculus), RATHSPA (M11942, Rattus norvegicus), HUMHSC70 (Y00371, Homo sapiens) and HAMHSP70A (M34561, Cricetulus griseus). Heat inducible HSP70 genes are as follows; MUSHSP70A (M19141, Mus musculus), CHKHSP (J02579, Gallus gallus) and XELHSP70B (M11915, Xenopus laevis). The hsp70 gene [TRCHSP7D(X13890)] of Trypanosoma cruzi is used for an outer group. Accession numbers and species name are shown in parenthesis. Numbers at each branch indicates the percentage of times a node was supported in 500 bootstrap pseudoreplication by neighbor joining.

members of HSP70 family from seven species of vertebrates. MUSHSPCA (Giebel et al., 1988), RATHSPA (O'Malley et al., 1985), HUMHSC70 (Dwornizak and Mirault, 1987), HAMHSP70A (Ahmad et al., 1990) and OLHSC70 formed a clade, excluding CHKHSP (Morimoto et al., 1986), MUSHSP70A (Zakeri et al., 1988) and XELHSP70B (Bienz, 1984) and TRCHSP7D (Requena et al., 1988) as an outgroup. It was reported that MUSHSPA, RATHSPA, HUMHSC70 and HAMHSP70A were expressed constitutively and MUSHSP70A, CHKHSP and XELHSP70B were induced by hyperthermia. This result indicated OLHSC70 was more closely related to hsc70 (cognate of hsp70) than to hsp70.

To analyze the expression of hsp70-related genes, the accumulation of heat-inducible transcripts after various heat-shock treatments (38 - 43°C) was examined by RNA dot-blots analysis with probe A.. We found that the accumulation of heat-inducible transcripts increased dramatically after heat-shock treatment at 41°C in OL32 cells and at 42°C in CE cells (Arai et al., 1994). This result indicates that the pattern of expression of hsp70 related genes differed between OL32 cells and CE cells at the transcriptional level (Arai et al., 1994).

To analyze the transcription of the gene corresponded to the OLHSC70 cDNA, we
isolated total RNA from OL32 cells 1 h and 24 h after heat-shock treatment, and subjected it to dot-blot analysis using probes A and B. Probe A detected heat-inducible transcripts 1 h after heat shock, and the level of transcripts decreased within 24 h after heat shock (Fig. 3). The transcripts detected by probe B were found in both heat-shocked and non-heat-shocked cells and the increase in levels of transcripts caused by heat treatment were not so dramatic (Fig. 3). This result indicates that the most of the transcripts detected by probe B were not heat-inducible and that the gene corresponding to OLHSC70 is a constitutively expressed hsp70-related gene.

![Fig. 3. Identification of transcripts after heat-shock treatment of medaka cultured cells.](image)

Two-dimensional electrophoresis of proteins shows that OL32 cells contained at least three heat-inducible proteins with molecular masses of 70 kDa (Fig. 4 a,b). One (Hsp71.1) was synthesized at a low level at 33°C and was induced immediately after heat-shock treatment (41°C), returning to a normal level within 24 h after heat treatment (Arai et al. 1994). The remaining two proteins were constitutionally synthesized at 33°C but slightly enhanced levels were induced by heat-shock treatment and the proteins continued to be synthesized for a long time (Hsc70.1, Hsc70.2). OL32 cells transfected with pcOLHSC70 synthesized more Hsc70.1 than non-transfected control OL32 cells (Fig. 4 c). This result showed that Hsc70.1 encoded by pcOLHSC70 was expressed in OL32 cells. The genes corresponding to OLHSC70 cDNA and presumably to CEHSC70 cDNA appear to be members of the family of constitutively expressed hsp70 genes and the product of OLHSC70 is Hsc70.1.
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