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

Ayami ARAI, Kiyoshi NARUSE*, Hiroshi MITANI and Akihiro SHIMA

Laboratory of Radiation Biology, Department of Biological Sciences, School of Science, University of Tokyo, Bunkyo-Ku, Tokyo 113, Japan

(Received 20 February 1995)

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 hyperthermia. The other two proteins (Hsc70.1, and Hsc70.2) were constitutively expressed under normal conditions and only slightly enhanced levels were induced by hyperthermia. 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 thermotolerance and are associated with the thermosensitivity 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

* Corresponding author.

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 60 µg/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 8×10^7 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, GAAAGGCCAGTGCTTCATGTC; and P4, CTTTCATCAGGATTTATGCTCTTGTT.

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 (CGATTTGATGATCATGTTGTACAGT) 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 μ l of 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 0.2 mM deoxynucleoside triphosphates, 10 pM each primer, 1 μ g 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 micro-scale sample filtration manifold device. Ten μ g 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 $\times$ SSPE, 5% Irish Cream Liqueur (Baileys), 10 μ g/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 $\times$ SSC that contained 1% SDS at room temperature for one min, and twice with 2 $\times$ 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, pcDNA1 (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 10^8 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

of expression of HSP70 proteins in OL32 cells with or without transfection with pcOLHSC70 (OLHSC70 cDNA ligated to the eukaryotic expression vector pcDNA I) by two-dimensional electrophoresis.

Figure 2 shows a phylogenetic tree constructed from protein sequences of

1	G	GAA	TTC	GGC	ACG	AGG	TTT	TTT	TTT	TTT	ACT	CTC	CTC	GTC	CCG	GTA	ACG	TCG	CAT	TTG	58
59	CCC	CTC	CGT	TGT	CTG	CGG	TTT	CCA	AAC	ACA	CAC	TTT	CGG	CCA	CCG	ACC	ATC	TAT	TTT	GGA	118
(A)																					
(B) 119	AGC	ATG	TCT	AAG	GGA	CCA	GCA	GTC	GGC	ATT	GAT	CTA	GGA	ACC	ACC	TAC	TCC	TGT	GTT	GGT	178
(C) 1	M	S	K	G	P	A	V	G	I	D	L	G	T	T	Y	S	C	V	G		19
					A																
179	GTG	TTC	CAG	CAT	GGC	AAG	GTT	GAA	ATC	ATT	GCC	AAT	GAT	CAG	GGT	AAT	AGG	ACC	ACA	CCA	238
20	V	F	Q	H	G	K	V	E	I	I	A	N	D	Q	G	N	R	T	T	P	39
239	AGT	TAT	GTG	GCC	TTC	ACC	GAT	ACA	GAG	AGG	CTG	ATC	GGA	GAT	GCA	GCC	AAA	AAC	CAG	GTG	298
40	S	Y	V	A	F	T	D	T	E	R	L	I	G	D	A	A	K	N	Q	V	59
299	GCA	ATG	AAC	CCC	ACC	AAC	ACC	GTT	TTT	GAT	GCC	AAG	AGA	CTC	ATT	GGG	CGA	CGA	TTT	GAT	358
60	A	M	N	P	T	N	T	V	F	D	A	K	R	L	I	G	R	R	F	D	79
359	GAT	CAT	GTT	GTA	CAG	TCT	GAC	ATG	AAC	GAT	TGG	CCT	TTT	AAT	GTA	ATC	AAT	GAC	AAC	ACC	418
80	D	H	V	V	Q	S	D	M	N/K	D/H	W	P	F	N/A	V	I	N	D	N	T/S	99
419	CGT	CCA	AAG	GTC	CAG	GTT	GAA	TAC	AAA	GGA	GAA	ACA	AAG	TCC	TTC	TAC	CCT	GAG	GAG	GTT	478
100	R	P	K	V	Q	V	E	Y	K	G	E	T	K	S/P	F	Y	P	E	E	V/I	119
479	TCT	TCA	ATG	GTG	TTG	ACA	AAA	ATG	AAG	GAG	ATT	GCA	GAA	GCC	TAC	CTT	GGA	AAA	ACG	GTG	538
120	S	S	M	V	L	T	K	M	K	E	I	A	E	A	Y	L	G	K	T	V	139
539	AAC	AAT	GCT	GTT	ATC	ACA	GTA	CCA	GCC	TAC	TTC	AAT	GAC	TCC	CAA	CGT	CAG	GCA	ACC	AAG	598
140	N	N	A	V	I	T	V	P	A	Y	F	N	D	S	Q	R	Q	A	T	K	159
599	GAT	GCA	GGC	ACA	ATC	TCT	GGT	CTT	AAT	GTT	CTT	CGC	ATC	ATC	AAT	GAA	CCA	ACT	GCT	GCT	658
160	D	A	G	T	I	S	G	L	N	V	L	R	I	I	N	E	P	T	A	A	179
659	GCA	ATT	GCC	TAT	GGG	CTG	GAC	AAA	AAG	GTT	GGC	TCC	GAG	AGG	AAT	GTT	CTT	ATC	TTT	GAT	718
180	A	I	A	Y	G	L	D	K	K	V	G	S	E	R	N	V	L	I	F	D	199
719	CTT	GGT	GGT	GGC	ACC	TTT	GAT	GTT	TCC	ATC	TTG	ACA	ATT	GAG	GAT	GGT	ATT	TTT	GAG	GTC	778
200	L	G	G/A	G	T/I	F	D	V	S	I	L	T	I	E	D	G	I	F	E	V	219
779	AAG	TCT	ACT	GCT	GGA	GAC	ACT	CAT	CTT	GGT	GGT	GAA	GAT	TTT	GAC	AAC	CGC	ATG	GTT	AAT	838
220	K	S	T	A	G	D	T	H/N	L	G	G	E/G	D	F	D	N	R	M	V	N	239
839	CAC	TTC	ATC	GCA	GAG	TTT	AAA	CGC	AAA	TAC	AAG	AAA	GAC	ATC	AGT	GAC	AAC	AAG	AGG	GCT	898
240	H	F	I	A	E/X	F/X	K	R	K	Y	K/X	K	D	I	S	D	N	K	R	A	259
899	GTC	CGC	CGC	CTG	CGC	TCT	GCT	TGT	GAG	AGG	GCC	AAG	CGA	ACA	CTG	TCT	TCC	AGC	ACT	CAA	958
260	V	R	R	L	R	S/T	A	C	E	R	A	K	R	T	L	S	S	S	T	Q/X	279
959	GCA	AGT	ATT	GAA	ATT	GAT	TCC	TTG	TAC	GAG	GGA	GTC	GAC	TTT	TAT	ACG	TCC	ATC	ACC	AGG	1018
280	A	S	I	E	I	D	S/P	L	Y	E	G	V/X	D/X	F	Y	T	S	I	T	R	299
1019	GCA	CGT	TTT	GAG	GAG	CTT	AAC	GCA	GAC	CTT	TTC	CGA	GGC	ACC	CTT	GAT	CCT	GTG	GAG	AAG	1078
300	A	R	F	E	E	L	N	A	D	L	F/X	R/X	G	T	L	D	P	V	E	K	319
1079	TCA	CTT	CGT	GAT	GCT	AAG	ATG	GAT	AAA	GGT	CAG	ATT	CAT	GAC	ATT	GTT	TTA	GTT	GGT	GGC	1138
320	S/V	L	R	D	A	K	M	D	K	G	Q	I	H	D	I	V	L	V	G	G	339
1139	TCC	ACT	CGC	ATC	CCG	AAG	ATC	CAA	AAA	CTA	CTC	CAA	GAC	TTC	TTT	AAT	GGA	AAA	GAG	CTC	1198
340	S	T	R	I	P	K	I/X	Q/X	K	L	L	Q/X	D/A	F/X	F	N	G	K	E/X	L	359

		P4	N N A	G	G C					T	C										
1199	AAC	AAA	AGC	ATC	AAT	CCT	GAT	GAA	GCG	GTG	GCT	TAT	GGA	GCT	GCT	GTG	CAG	GCT	GCG	ATC	1258
360	N	K	S	I/X	N/X	P	D	E	A/P	V	A	Y	G	A	A	V	Q	A	A	I	379
									T G			T					T				
1259	CTG	TCT	GGA	GAC	AAG	TCT	GAG	AAT	GTC	CAA	GAC	TTG	CTG	CTT	CTG	GAT	GTC	ACT	CCA	CTT	1318
380	L	S	G	D	K	S	E	N	V	Q	D	L	L	L	L	D	V	T	P	L	399
1319	TCT	CTG	GGT	ATA	GAG	ACT	GCT	GGT	GGG	GTG	ATG	ACA	GTC	TTA	ATC	AAA	CGC	AAT	ACT	ACA	1378
400	S	L	G	I	E	T	A	G	G	V	M	T	V	L	I	K	R	N	T	T	419
									C	G		G	C								
1379	ATT	CCT	ACC	AAA	CAA	ACC	CAG	ACC	TTT	ACT	ACA	TAC	TCT	GAT	AAC	CAG	CCT	GGG	GTG	CTC	1438
420	I	P	T	K	Q	T	Q	T	F	T	T	Y	S	D	N	Q	P	G	V	L	439
									GGT	A	C					T					
1439	AAC	CAG	GTT	TAT	GAA	---	GTG	GGT	GCC	ATG	ACC	AAG	GAC	AAC	AAC	CTG	CTG	GGA	AAG	TTT	1498
440	N	Q	V	Y	E		V/E	G/R	A	M	T	K	D	N	N	L	L	G	K	F	459
							A													T	
1499	GAA	CTG	ACA	GGA	ATT	CCT	CCT	GCT	CTC	TGG	TGT	CCT	CAG	ATT	GAG	GTG	ACA	TTT	GAT	ATT	1558
	E	L	T	G	I	P	P	A	L	W	C	P	Q	I	E	V	T	F	D	I	479
																				G	
1559	GAC	GCA	AAT	GGA	ATC	ATG	AAT	GTT	TCT	GCT	GTT	GAT	AAG	AGC	ACT	GGC	AAG	GAA	AAC	AAA	1618
	D	A	N	G	I	M	N	V	S	A	V	D	K	S	T	G	K	E	N	K	499
																				G	
1619	ATC	ACT	ATC	ACT	AAT	GAC	AAA	GGT	CGT	CTT	AGT	AAA	GAG	GAC	ATT	GAG	CGC	ATG	GTG	CAA	1678
500	T	I	T	N	D	K	G	R	L	S	K	E	D	I	E	R	M	V	Q	E	519
																				T	
1679	GAA	GCT	GAG	AAG	TAC	AAG	GCA	GAA	GAT	GAT	GTT	CAG	CGT	GAC	AAA	GTG	TCT	GCA	AAG	AAT	1738
	E	A	E	K	Y	K	A	E	D	D	V	Q	R	D	K	V	S	A	K	N	539
																				G C A	
1739	GGC	CTG	GAG	TCC	TAT	GCT	TTC	AAC	ATG	AAG	TCA	ACT	GTG	GAG	GAT	GAG	AAG	CTT	GCT	GGC	1798
540	G	L	E	S	Y	A	F	N	M	K	S	T	V	E	D	E	K	L	A	G	559
																				G	
1799	AAG	ATC	AGT	GAT	GAA	GAC	AAA	CAA	AAG	ATC	TTG	GAT	AAG	TGC	AAT	GAA	GTG	ATC	AGC	TGG	1858
560	K	I	S	D	E	D	K	Q	K	I	L	D	K	C	N	E	V	I	S	W	579
																				A C T A T	
1859	CTG	GAC	AAG	AAC	CAG	ACT	GCT	GAA	AGA	GAT	GAG	TAC	GTG	CAC	CAA	CAG	AAG	GAG	CTT	GAG	1918
580	L	D	K	N	Q	T	A	E	R/K	D	E	Y	V/E	H	Q	Q	K	E	L	E	599
																				T	
1919	AAA	GTA	TGT	AAT	CCC	ATC	ATC	ACC	AAA	CTG	TAC	CAG	AGT	GCT	GGG	GGC	ATG	CCT	GGA	GGA	1978
	K	V	C	N	P	I	I	T	K	L	Y	Q	S	A	G	G	M	P	G	G	619
																				C	
1979	TGC	CAG	AGG	GAA	TGC	CAG	GAG	GTT	TCC	CTG	GAG	CTG	GTG	GTG	CTG	---	CTG	GCG	GTG	GCT	2038
	C	Q	R	E	C	Q	E	V	S	L	E	L/T	V	V	L	L	A	V	A/E	L/D	639
																				C G A G C	
2039	CTT	CTG	GAC	CAA	CCA	TTG	AAG	AAG	TTG	ATT	AAA	CAT	TCC	ATG	ACT	TCA	CCT	TCT	AAG	ATC	2098
	L	L	D	Q	P	L	K	K	L	I/T	K/R	H	S	M/K	T/A	S	P	S	K	I	659
																				TCT	
2099	TTT	ACT	---	---	AAG	AAA	ACC	CTC	TTT	AAG	TGC	AAT	ATG	CAA	TCT	ATA	AAG	AGT	GTA	ATG	
	F	T			K	K	T	L	F	K	C	N	M	Q	S	I	K	S	V	M	

2159	AGG	TTA	AAC	CAG	TTT	CCT	TTT	ATT	ACC	ATT	ACT	ZZA	AGG	ACG	AGT	ACA	ACT	TTG	TCA	CTT	
	R	L	N	Q	F	P	F	I	T	I	T										
																				T	
2219	CCT	ATG	GGG	TGA	TTT	ACA	TAT	TTG	GAT	GTC	ACT	CTA	CAA	TAA	ATG	CTG	AAA	CCA	C	2261	

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.

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(X13690)] 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 TRCHSP70D (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-blot 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. Cells were heat-shocked at 33°C or 41°C for 1 h, and then incubated at 33°C for 1 h or 24 h. Total RNA was isolated with a RNA Extraction Kit (Pharmacia). Ten μ g of RNA were blotted in each slot. Probe A was the product of PCR with primers P1 and P2 (see Fig. 1). Probe B was a fragment obtained by digestion with PvuII, that contained the 3' non-coding region of OLHSC70 and part of the vector sequence.

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 constitutively 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.

Fig. 4. Two-dimensional electrophoresis of proteins from cultured medaka cells (OL32). A: Non-heat-shocked and non-transfected control cells. B: Heat-shocked cells incubated at 41°C for 1 h and then at 33°C for 1 h. C: Cells transfected with pcOLHSC70. a, Hsc70.1; b, Hsc70.2; c, Hsp70.1; d, Hsp90.

This research was supported by a grant to A.S. from the Fisheries Agency, Japan and in part by Grant-in-Aid for Scientific Research on Priority Areas to H.M..

REFERENCES

Ahmad, S., Ahuja, R., Venner, T. J. and Gupta, R. S. (1990). Identification of a protein altered in

- mutants resistant to microtubule inhibitors as a member of the major heat shock protein (hsp70) family. *Mol. Cell. Biol.* **10**, 5160–5165.
- Arai, A., Mitani, H., Naruse, K., and Shima, A. (1994). Relationship between the induction of proteins in the HSP70 family and thermosensitivity in two species of *Oryzias* (Pisces). *Comp. Biochem. Physiol.* **109B**, 647–654.
- Ashburner, M. and Bonner, J. J. (1979). The induction of gene activity in *Drosophila* by heat shock. *Cell* **17**, 241–254.
- Bienz, M. (1984). *Xenopus* hsp70 genes are constitutively expressed in injected oocytes. *EMBO J.* **3**, 2477–2483.
- Dworniczak, B. and Mirault, M. E. (1987). Structure and expression of a human gene coding for a 71 kd heat shock ‘cognate’ protein. *Nucleic Acids Res.* **15**, 5181–5197.
- Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6–13.
- Giebel, L. B., Dworniczak, B. P. and Bautz, E. K. F. (1988). Developmental regulation of a constitutively expressed mouse mRNA encoding a 72-kDa heat shock-like protein. *Dev. Biol.* **125**, 200–207 (1988).
- Hayasaka, K., Sato, M., Mitani, H. and Shima, A. (1990). Transfection of cultured fish cells RBCF-1 with exogenous oncogene and their resistance to malignant transformation. *Comp. Biochem. Physiol.* **96B**, 349–354.
- Higgins, D. G. and Sharp, P. M. (1988). CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. *Gene* **73**, 237–244.
- Hunt, C. and Calderwood, S. (1990). Characterization and sequence of a mouse hsp70 gene and its expression in mouse cell lines. *Gene* **87**, 199–204.
- Hunt, C. and Morimoto, R. (1985). Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. *Proc. Natl. Acad. Sci. USA* **82**, 6455–6459.
- Innis, M. A., Gelfand, D. H., Sininsky, J. J. and White, T. J. (1990). *PCR protocol*. Academic Press, San Diego, Calif.
- Komura, J., Mitani, H. and Shima, A. (1988). Fish cell culture: establishment of two fibroblast-like cell lines (OL-17 and OL-32) from fins of the Medaka, *Oryzias latipes*. *In Vitro Cell. & Devel. Biol.* **24**, 294–298.
- Kumar, S., Tamura, K., and Nei, M. (1993). *MEGA: Molecular Evolutionary Genetic Analysis Version 1*. The Pennsylvania State University, University Park, Pa.
- Morimoto, R. I. (1990). *Stress proteins in biology and medicine*. Cold Spring Harbor Laboratory Cold Spring Harbor, N.Y.
- Morimoto, R. I., Hunt, C., Huang, S.-Y., Berg, K. L. and Banerji, S. S. (1986). Organization, nucleotide sequence and transcription of the chicken HSP70 gene. *J. Biol. Chem.* **261**, 12692–12699.
- Naruse K., Shima A., Matsuda M., Sakaizumi M., Iwamatsu T., Soeroto B., and Uwa H. (1993). Distribution and phylogeny of rice fish and their relatives belonging to the suborder Adrianichthyoidei in Sulawesi, Indonesia. *The Fish Biol. J. Medaka* **5**, 11–15.
- Oda, S., Mitani, H., Naruse, K. and Shima, A. (1991). Synthesis of heat shock proteins in the isolated fin of the Medaka, *Oryzias latipes*, acclimatized to various temperatures. *Comp. Biochem. Physiol.* **98B**, 587–591.
- O’Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007–4021.
- Ohtsuka K. and Laszlo A. (1992). The relationship between hsp70 localization and heat resistance. *Exp. Cell Res.* **202**, 507–518.
- O’Malley, K., Mauron, A., Barchas, J. D. and Kedes, L. (1985). Constitutively expressed rat mRNA

- encoding a 70-kilodalton heat-shock-like protein. *Mol. Cell. Biol.* **5**, 3476–3483.
- Requena, J. M., Lopez, M. C., Jimenez-Ruiz, A. and Alonso, C. (1988) A head to tail tandem organization of hsp70 genes in *T. cruzi*. *Nucleic Acids Res.* **16**, 1393–1406.
- Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for constructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425
- Schlesinger, M. J., Ashburner, M. and Tissieres, A. (1982). *Heat Shock: From Bacteria to Man*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Subiec, J., Sciandra, J. J. and Johnson, R. J. (1982). Heat shock proteins and thermotolerance; a comparison of induction kinetics. *Br. J. Radiol.* **55**, 579–584.
- Wang, V., Naruse, K. and Phang, S. H. (1993). Mini iso-electric focusing device to be used with the Bio-Rad Mini-Protean II sysyem. *Anal. Biochem.* **212**, 299–301.
- Zakeri, Z. F., Wolgemuth, D. J. and Hunt, C. R. (1988). Identification and sequence analysis of a new member of the mouse HSP70 gene family and characterization of its unique cellular and developmental pattern of expression in the male germ line. *Mol. Cell. Biol.* **8**, 2925–2932.