Complementary DNA Cloning of HSC71, a 71 kDa Heat Shock Cognate Protein, in Japanese Flounder *Paralichthys olivaceus*

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Full-length cDNA for a 71 kDa constitutively expressed heat-shock-cognate protein (HSC71), a member of the HSP70 family, was isolated from a Japanese flounder embryo cDNA library. It has a single ORF of 1950bp that encodes a protein of 71.2 kDa. Japanese flounder HSC71 contains a EEVD (Glu-Glu-Val-Asp) peptide motif at the C-terminal end which is a common feature of cytosolic HSC proteins in the HSP70 family. Japanese flounder HSC71 is 96.0%, 93.9%, 93.5%, and 93.4% identical in primary structure to rainbow trout HSC71, bovine HSC71, rat HSC71, and human HSC71, respectively. These results suggest that the HSC71 amino acid sequence has been highly conserved among vertebrates. Northern blot analysis showed that HSC71 mRNA was expressed constitutively in the Japanese flounder cells in early passage regardless of heat shock treatment.

Key words: HSC71, heat shock cognate protein, cDNA, Japanese flounder

All organisms synthesize a few evolutionary-conserved proteins called heat shock proteins or HSP, recently called a stress protein, in response to various harmful environmental stresses.¹⁾ The most abundant and conserved HSP has a molecular mass of about 70 kDa. In eukaryotes, HSP70 are encoded by heat-inducible HSP70 genes and usually their basal expression is negligible. Several homologous were subsequently discovered, which were constitutively expressed under normal and stress conditions in contrast to HSP70.¹⁾ One of these so-called heat shock cognate genes encodes about 70 kDa protein (HSC70) which is particularly abundant in embryos and ovaries.²⁾ In yeast, genetic analysis has shown that double mutations into two cognate genes affected the growth rate at normal temperature, but not at a high temperature which induces the expression of HSP70,³⁾ suggesting that HSC70 is essential for normal growth. Although the patterns in expression of HSP and HSC are different, it was shown that the HSC70 and HSP70 have similar structure and function as molecular chaperones in cultured cells of mammals.⁴⁾ These findings suggest that HSC70 is indispensable for the development of fish embryos under normal and stress conditions.

Japanese flounder is one of the most economically important fish in Japan. The cultivation of Japanese flounder has been successful. During the transportation and breeding in the farm, the embryos could be exposed to adverse environmental changes. Therefore, stress proteins includ-

ing HSC70 may have important roles for the development under normal and stress conditions. In fact, in vitro studies revealed that the cultured fish cells constitutively synthesized HSC70 under normal conditions and adverse conditions such as heat shock, heavy metal ions, and sodium arsenite.¹⁾ However, little is known about such proteins and those gene structures in Japanese flounder. As a preliminary experiment to resolve the role of stress protein in the flounder embryos, we decided to clone the HSC70-related cDNA, referred to here as Japanese flounder HSC71 cDNA. Nucleotide sequence and expression analyses revealed that the isolated clone represents a heat shock cognate 70 cDNA.

Materials and Methods

Japanese Flounder Embryos and Culture of cells

Japanese flounder *Paralichthys olivaceus* embryos in the eyed period were kindly supplied by Yamagataya (Nantan, Hyogo, Japan). Primary cultured cells of Japanese flounder embryos were kindly donated by Dr. T. Seikai (Fisheries Research Station, Faculty of Agriculture, Kyoto University). The cells were cultured at 25°C in air using Leibovitz's L15 medium supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, penicillin potassium (200 international units/ml), streptomycin sulfate (200 µg/ml), and 10% fetal bovine serum (Biological

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The following abbreviations are used: HSC, heat shock cognate protein(s); HSP, heat shock protein(s); RT-PCR, reverse transcription-polymerase chain reaction; cDNA, DNA complementary to mRNA; bp, base pair; kb, kilobase; dNTP, deoxynucleotide triphosphate; SDS, sodium dodecyl sulphate; SSC, standard sodium chloride/sodium citrate buffer

The DDBJ, GenBank and EMBL accession number for Japanese flounder HSC71 is AB006814.

Industries).

Preparation of RNA

Total RNA from embryos of Japanese flounder or the cultured cells in early passage from flounder embryo was isolated by ultracentrifugation in 5.7 M cesium chloride after homogenization in 4 M guanidinium thiocyanate.⁵⁾ Poly (A)+ RNA was enriched by chromatography on oligo (dT) cellulose (Collaborative Biomedical Products) or on Oligotex-dT30 (Takara).

RT-PCR Cloning

To obtain a partial sequence of flounder HSC71 gene, PCR was conducted with degenerate oligonucleotide primers having the following sequences: 5'-ATCGAYCTSGGSACYACCTACTC-3' as the sense primer and 5'-GCACCGTASSCSACCGCYTCRTC-3' as the an-

tisense primer, where R=A+G, S=G+C, Y=C+T. These correspond to the amino acid sequences IDLGT-TYS and DEAVAYGA, respectively, which are identical in rainbow trout HSC71,⁶⁾ human HSC71,⁷⁾ (shown in Fig. 1), bovine HSC71,⁸⁾ and rat HSC71.⁹⁾ Single-stranded cDNA was synthesized from 1 µg of total RNA of the embryo with 200 units of MMLV-RT (Gibco-BRL) in a 20 µl reaction mixture comprising 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 0.1 mg/ml BSA, 1 mM each dNTP, 5 µM random hexamer, and 20 units of ribonuclease inhibitor RNasin (Toyobo). A quarter of the resultant cDNA was used as a template for the PCR, which was carried out in a 25 µl solution comprising 8 units of ΔTth DNA polymerase (Toyobo), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 0.1 mg/ml BSA, 0.2 mM each dNTP, and 0.2 µM each primer. The PCR conditions were 30 s at 95°C, 1 min at 56°C, and 2 min at

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-61                                     gacaagtcgcctcaccgcacccaacacctaagtcacgcggttcaattatccggaatc -1
  M S K G P A V G I D L G T T Y S C V G V F Q H G K V E I I A N D Q
1  ATGTCTAAGGACCAGCAGTGGTATTGATCTCGGGACCACCTACTCTGTGGTGTGTTCCAGCATGAAAAGTTGAGATCATTGCCAATGACCAGG 100
  G N R T T P S Y V A F T D S E R L I G D A A K N Q V A M N P T N T V
101 GCAACAGGACCACACCCAGTATGTGGCCTTACAGATTCCGAGAGGCTGATTGGAGATGCCAAAAACAGGTTGCCATGAACCCACCAACACGGT 200
  F D A K R L I G R R F D D A V V Q S D M K H W P F N V I N D S T R
201 CTTTGATGCAAAAAGGCTGATCGGCCGAGGTTGATGACGAGTGTACAATCAGATATGAAGCACTGGCCATTCAATGTCAATGACAGCACTCGC 300
  P K V Q V E Y K G E S K S F Y P E E I S S M V L T K M K E I A E A
301 CCCAAAGTTCAAGTTGAGTACAAGGGTGAGTCAAAGTCTCTACCCAGAGGAGATCTCATCATGGTGCTGACCAAGATGAAGGAGATTGCTGAAGCCT 400
  Y L G K T V N N A V V T V P A Y F N D S Q R Q A T K D A G T I S G L
401 ACCTCGGAAAGACTGTCAACAATGCTGTAGTCACAGTGCCCGCTACTTCAATGACTCCAGCGCCAGGCCACCAAGGATGCAGGCACTATCTGGCCT 500
  N V L R I I N E P T A A A I A Y G L D K K V G S E R N V L I F D L
501 CAATGTTTTGCGTATCATCAATGAACCGACTGCTGCTGCCATCGCTATGGTTTGGACAAGAAGTTGGATCAGAAAGAACGCTCTCATCTTCGATCTT 600
  G G G T F D V S I L T I E D G I F E V K S T A G D T H L G G E D F
601 GGTGTGGCACCTTGTATGTGTCCATCTTGACCATGAGGATGGCATCTTGAAGTCAAGTCCACCGCTGGAGATACTCATCTTGGTGGGAAGATTTG 700
  D N R M V N H F I A E F K R K Y K K D I S D N K R A V R R L R T A C
701 ACAACCGCATGGTCAACCACTTCATCGCTGAGTTCAAGCGCAAGTACAAGAAAGACATCAGCGACAACAAGAGAGCTGCTCGTCTGCGCCAGCGCTTG 800
  E R A K R T L S S S T Q A S I E I D S L Y E G V D F Y T S I T R A
801 TGAGAGGGCAAGCGCATTTGCTTCCAGCAGCCAGGCCAGCATCGAAATCGACTCCCTGTATGAGGGAGTTGACTTTTACACCTCCATCACCAGGGCT 900
  R F E E L N A D L F R G T L D P V E K S L R D A K M D K G Q I H D
901 CGCTTTGAGGAGCTCAATGCTGACCTCTTCCGTGGCACTAGACCTGTGGAGAAGTCTCTCCGTGATGCCAAGATGGATAAGGGACAGATCCATGACA 1000
  I V L V G G S T R I P K I Q K L L Q D F F N G K E L N K S I N P D E
1001 TTGCTCTGGTGGTGGCTCCACCGTATCCCCAAGATCCAGAAGCTGCTCCAGGACTTCTTCAACGGAAGAGCTCAACAAGAGTATCAACCTGATGA 1100
  A V A Y G A A V Q A A I L S G D K S E N V Q D L L L L D V T P L S
1101 AGCTGTGGCTATGGAGCGCTGTCCAGGCTGCCATCTGTCTGGTGACAAGTCTGAAATGTGAGGACCTGCTGCTTCTGGATGTACCCCCCTGTCC 1200
  L G I E T A G G V M T V L I K R N T T I P T K Q T Q T F T T Y S D
1201 CTGGGTATTGAGACTGTGGAGGCGTCATGACTGTCTTGATCAACGCAACACCACCATCTCTACTAAGCAGACCCAGACCTTTACCACCTACTCTGACA 1300
  N Q P G V L I Q V Y E G E R A M T R D N N L L G K F E L T G I P P A
1301 ACCAGCTGGTGTGCTCATCAGGTTTATGAGGGTGAGCGTGCCATGACCAGAGACAACAACCTGCTGGGCAAGTTTGAAGTACAGGCGATCCCTCTGTC 1400
  P R G V P Q I E V T F D I D A N G I M N V S A A D K S T G K E N K
1401 TCCTCGTGGTGTTCCTCAGATCGAGGTGACATTTGATATCGATGCCAACGGCATCATGAATGTCTCTGCTGCTGACAAGAGCACTGGAAGGAGAACAAG 1500
  I T I T N D K G R L S K E D I E R M V Q E A E K Y K T E D D V Q R
1501 ATCACCATACCAATGACAAGGGTCTCTCAGCAAGGAGGACATTGAACGTCATGGTCCAGGAGGCTGAGAAGTACAAGACAGAGGATGATGTCCAGCGTG 1600
  D K V S A K N G V E S Y A F N M K S T V E D E K L A G K I S D E D K
1601 ACAAGGTGTCTGCAAGAATGGAGTGGAGTCATATGCTTTCAACATGAAGTCAACTGTGGAGGATGAGAAGCTCGTGGCAAGATCAGTGATGAAGATAA 1700
  Q K I L D K C N E V I S W L D K N Q T A E K D E Y E H Q Q K E L E
1701 GCAGAAGATCTTGACAAGTGAACGAGGTTCATGAGTGGCTGGACAAGAACAGACTCGCGAAAAGGATGAATATGAGCATCAACAAAAGGAAGTGGAG 1800
  K V C N P I I T K L Y Q S A G G M P G G M P E G M P G G F G G A G
1801 AAGGTGTGCAACCCCATCATCACTAAGCTGTACCAGAGTCCCGGTGGCATGCCAGGTGGTATGCCAGAAGGCATGCCTGGTGGCTTCGGTGGAGCTGGTG 1900
  G A A P G A G S S G P T I E E V D
1901 GTGCCGCTCTGGCGCTGGATCATCTGGCCCCACCATGAGGAGGTGCAGTaaacattccctcagctgctacctcctgagaaagtgtctaaagaagtaa 2000
2001 ccctcttatagcacaagttacaaagcttaagagtgtaatttaagatcttgttattgcatcatttgacacaaactgggaacagcttgacttggtcagtg 2100
2101 gagcagtgagtcacaaagcggtttgtatgatgtcactgcctgtgcataaagcatgaacccctcaaaaaaaaaaaaaaaaaa 2181

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Fig. 1. Nucleotide and deduced amino acid sequences of the HSC71 cDNA from Japanese flounder embryos.

Small letters denote the 5' and 3' untranslated regions and capital letters denote the coding regions. The position of initiation (base pairs 1 to 3), termination (1951 to 1953) codons, *EcoRV* (1435 to 1440) and *HindIII* (2022–2027) sites, and polyadenylation signal (2146 to 2151) in the sequence are underlined.

72°C for 25 cycles (Astec, Program Temp Control System PC-700). The resultant products of the expected size, approximately 1090 bp, were subcloned in pBluescript II KS- (Stratagene). DNA sequencing analysis revealed that there was a cDNA fragment of HSC71-like gene in the pool of the PCR products.

cDNA Cloning and Sequencing

The synthesis of double-stranded cDNA was accomplished with a ZAP-cDNA Synthesis Kit (Stratagene) using poly (A)+ RNA prepared from embryo by chromatography on oligo (dT) cellulose. The cDNAs were size-fractionated on a CHROMA SPIN-100 column (Clontech) and then adapter-ligated prior to the generation of an oligo (dT) primed library in Uni-ZAP/*Eco*RI/*Xho*I/*CIAP* (Stratagene). The cDNA library was screened with a

cDNA fragment obtained on PCR as a probe. Plaque hybridization was performed at 42°C in a solution comprising 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS, 50% formamide, 100 mg/ml denatured salmon sperm DNA, 0.6% Ficoll 400, 0.6% polyvinylpyrrolidone 100, 0.6% BSA, and ³²P-labeled probe prepared with a Megaprime DNA labelling system (Amersham International PLC). After hybridization, the filters were washed four times with 2 × SSC at room temperature and three times with 2 × SSC, 0.5% SDS at 65°C prior to autoradiography. Plasmids (pBluescript SK-) containing the cDNA insert were obtained by *in vivo* excision from positive single plaques according to the manufacturer's instruction (Stratagene). After subcloning into pBluescript II KS+, DNA fragments were sequenced by a PCR procedure employing fluorescent dideoxynucleotides

fHSC71	1	MSKGPVAVGIDL GTTYS CVGVFQHGKVEIIANDQGNRTTSPYVAFTD SERLIGDAAKNQVAMNPTNTV FDAKRLIGRRFDD	80
tHSC71	1	C.....	80
hHSC71	1	T.....	80
hHSP70	1	.A.AA.I.....T.....L..Q.....K.G.	80
fHSC71	81	AVVQSDMKHWP FNVINDSTRPKVQVEYKGESKSFYEEISSMVLTKMKEIAEAYLGKTVNNAVVTVPAYFND SQRQATKD	160
tHSC71	81	G.....E.....L.....T.....V.....	160
hHSC71	81	M.V..AG.....T.....V.....T.....	160
hHSP70	81	P.....Q....GDK....S....T.A.....YP.T..I.....	160
fHSC71	161	AGTISGLNVLRIINEPTAAAIAYGLDKKVGSE RNVLI FDLGGGT F DVSILTIEDGIFEVKSTAGDTHLGGEDFDNRMVNH	240
tHSC71	161	A.....	240
hHSC71	161	...A.....A.....	240
hHSP70	161	..V.A.....RTGKG.....D.....A.....L....	240
fHSC71	241	FIAEFKRKYKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGVDFYTSITRARFEELNADLFRGTLDPVEKS	320
tHSC71	241	I.....	320
hHSC71	241	H.....E.....I.....A	320
hHSP70	241	.VE.....H....Q.....L....F..I.....CS...S..E...A	320
fHSC71	321	LRDAKMDKGQIHDIVLVGGSTRIPKIQKLLQDFNGKELNKSINPDEAVAYGA AVQAAILSGDKSENVQDLLLLDVTPLS	400
tHSC71	321	A.V.....	400
hHSC71	321	L..S.....	400
hHSP70	321	L..A...L.....V.....RD.....M.....A...	400
fHSC71	401	LG IETAGGVMTVLIKRNTTIPTKQTQTFTTYS DNQPGVL IQVYEGERAMTRDNNLLGKFELTGIPPAPRGVPQIEVTFDI	480
tHSC71	401	K.....	480
hHSC71	401	K.....	480
hHSP70	401	..L.....A....S.....I.....K.....R...S.....	480
fHSC71	481	DANGIMNVSAADKSTGKENKITITNDKGR LSKEDIERMVQAEKYKTEDDVQRDKVSAKNGVESYAFNMKSTVEDEKLAG	560
tHSC71	481	C.....S..SL.....Q.	560
hHSC71	481	L....V.....A..EK.....S..SL.....A.....Q.	560
hHSP70	481	L..T.T.....A.....E.....A..E...ER....AL.....A....G.K.	560
fHSC71	561	KISDEDKQKILDKCNEVISWLDKNQTAEKDEYEHQQKELEKVCNPIITKLYQSAGGMPGGMPEGMPGGFGGAGGAAPGAG	640
tHSC71	561	T...E.....G.....E.....H.....G.....A...P.....G.	640
hHSC71	561	..N.....I.N.....E.F.....P.....P.SG.	635
hHSP70	561	..EA..K.V....Q.....A.TL....F..KR....Q....SG...G...- - - - -Q.--KG.	630
fHSC71	641	-SSGPTIEEVD	650
tHSC71	641	G.....	651
hHSC71	561	A.....	645
hHSP70	631	SG.....	641

Fig. 2. Protein comparison.

The deduced amino acid sequence of a protein encoded by the cloned Japanese flounder cDNA (fHSC71) is compared to that of rainbow trout constitutively synthesized HSC71 (tHSC71),⁶ human HSC71 (hHSC71),⁷ and human heat-inducible HSP70 (hHSP70).¹⁰ Only different amino acids are indicated and hyphens mark deletions.

Fig. 3. RNA expression analysis.

Northern blot analyses were carried out using 10 μ g total RNA isolated from control (0 min) and heat-shocked (37°C for 20 or 40 min) Japanese flounder cells in early passage. The probe for hybridization was prepared from the cloned cDNA fragments (base pairs 1438 to 2026). Hybridization was done under the same conditions as described above. As an internal control, 28S rRNA on the filter was stained with methylene blue after hybridization and exposure to X-ray film.

and a model 373A automated sequencer (Applied Biosystems Inc.). The sequences reported in this paper were determined on both strands.

Northern Blot Analysis

The cultured cells from Japanese flounder embryos in early passage were heat-shocked at 37°C for 0, 20 or 40 min. Immediately after the treatment, the cells were collected. Northern analysis was carried out using total RNA (10 μ g) prepared from the cells. Cloned cDNA of Japanese flounder HSC71 was digested by *EcoRV* (base pairs 1435 to 1440) and *HindIII* (2022–2027) (Fig. 1). The cDNA fragment (1438 to 2026) was used as the probe for hybridization. Hybridization was performed under the same conditions as described above.

Staining RNA after Transfer to Filters

As an internal control, RNA on the filter was stained after hybridization and exposure to X-ray film. The dried filter was soaked and stained in a solution of 0.2 M sodium acetate (pH 5.2) and 0.2% methylene blue for 5 min at room temperature. The filter was rinsed in water for 10 min.

Results and Discussion

Cloning and Sequence of the Full-length cDNA for HSC71

An RT-PCR strategy for cloning a partial cDNA of Japanese flounder HSC71 successfully yielded a cDNA fragment of approximately 1090 bp. DNA sequencing analysis revealed that the fragment was homologous to rainbow trout HSC71 gene⁶ (data not shown). In order to obtain a full length cDNA of flounder HSC71, a flounder embryo cDNA library was screened with the PCR derived cDNA fragments as a probe. By screening of 10⁵ independent recombinant phages, seven positive clones were isolated. DNA sequencing analyses on both 5' and 3'-end revealed that they encoded the same gene (data not shown). As a result, we determined the nucleotide sequences of the

longest clone.

The cDNA of Japanese flounder HSC71 consists of 2242 nucleotides with poly (A) tail (Fig. 1). The in-frame stop codon in the 5' and 3' regions of the cDNA indicated that the clone contains the entire coding regions. The coding region includes 1950 nucleotides and corresponds to 650 amino acids with a predicted mass of 71.2 kDa.

Figure 2 compares the nucleotide sequence of HSC71 from Japanese flounder with those of other vertebrate. Japanese flounder HSC71 contains a EEVD (Glu-Glu-Val-Asp) peptide motif at the C-terminal end which is the common feature of cytosolic HSC proteins in the HSP70 family.¹⁾ Comparison with protein sequences in the SWISS-PROT database by FASTA indicated that Japanese flounder HSC71 is 96.0%, 93.9%, 93.5%, and 93.4% identical in primary structure to rainbow trout HSC71,⁶ bovine HSC71,⁸ rat HSC71,⁹ and human HSC71,⁷ respectively. Obviously the HSC71 amino acid sequence has been highly conserved in vertebrates. On the other hand, similarity to the heat inducible HSP70 is more divergent. For example, similarity to human HSP70¹⁰⁾ amounts to 84.5% (Fig. 2).

Constitutive Expression of Japanese Flounder HSC71 mRNA

To investigate the expression pattern of Japanese flounder HSC71 mRNA, Northern blot analyses of total RNA from normal or heat-shocked cells were conducted (Fig. 3). The probe for hybridization was prepared from the cloned cDNA fragments (base pairs 1438 to 2026) that contained 3' untranslated regions (Fig. 1) to tighten the specific affinity on HSC71 mRNA rather than HSP70 mRNA. An approximate 2.2-kb band was observed on RNA isolated from unheat shocked cells. No significant signal increase was observed after heat shock. Yamashita¹⁾ reported the similar results on the platyfish HSC70, and also Zefarullah *et al.* on the rainbow trout HSC71.⁶⁾ The structural similarities (Fig. 2) together with an identical mode of expression (Fig. 3) leave no doubts that the cloned cDNA described in this study represents a cDNA of heat shock cognate protein.

Analyses of the expression patterns of Japanese flounder HSC71 in embryos during development under normal and stress conditions are now in progress. cDNA cloning of Japanese flounder HSP70 and analyses of the expression patterns of HSP70 in embryos under normal and stress conditions are also now in progress.

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