| | |
|---|---|
| Title | マガキ HSC71 の cDNA クローニング<br>Complementary DNA Cloning of HSC71, a 71 kDa Heat Shock Cognate Protein, in Japanese Oyster Crassostrea gigas. |
| Author(s) | 横山　芳博（YOKOYAMA Yoshihiro） |
| *Citation* | 生活科学論叢（Review of Living Science），<br>No.33：77-84 |
| Issue Date | 2002 |
| Resource Type | Bulletin Paper / 紀要論文 |
| Resource Version | |
| URL | |
| Right | |
| Additional Information | |

# Complementary DNA Cloning of HSC 71, a 71 kDa Heat Shock Cognate Protein, in Japanese Oyster *Crassostrea gigas*

**YOKOYAMA Yoshihiro***

## ABSTRACT

Full-length cDNA for a 71 kDa constitutively expressed heat-shock-cognate protein (HSC 71), a member of the HSP 70 family, was isolated from a Japanese oyster cDNA library. It has a single ORF of 1977 bp that encodes a protein of about 71 kDa. Japanese oyster HSC 71 contains a EEVD (Glu-Glu-Val-Asp) peptide motif at C-terminal end which is a common feature of cytosolic HSC proteins in HSP 70 family. Japanese oyster HSC 71 is 87.7% and 88.9% identical in primary structure to Japanese flounder HSC 71 and human HSC 71, respectively. These results suggest that the HSC 71 amino acid sequence has been highly conserved among eukaryotes. Northern blot analysis showed that HSC 71 mRNA was expressed constitutively in the Japanese oyster tissues regardless of heat shock treatment.

**Key words** : HSC 71, heat shock cognate protein, cDNA, Japanese oyster.

---

*To whom correspondence should be addressed. E-mail : yokoyama@icis.shoin.ac.jp

The following abbreviations are used : HSC, heat-shock-cognate protein. MMLV-RT, moloney murine leukemia virus reverse transcriptase. RT-PCR, reverse transcription polymerase chain reaction. SSC, standard saline citrate.

## INTRODUCTION

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, HSP 70 are encoded by heat-inducible HSP 70 genes and usually their basal expression is negligible. Several homologs were subsequently discovered , which were constitutively expressed under normal and stress conditions in contrast to HSP 70.[1] One of these so-called heat shock cognate genes encodes about 70 kDa protein (HSC 70) 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 HSP 70,[3] suggesting that HSC 70 is essential for the normal growth. Although the patterns in expression of HSP and HSC are different, it was shown that the HSC 70 and HSP 70 have similar structure and function as molecular chaperons in cultured cells of mammals.[4] These findings suggest that the HSC 70 is indispensable for the cell-mediated life activities under normal and stress conditions.

Japanese oyster is one of the most economically important shellfish in Japan. Oyster is a sessile organism and could be exposed to adverse environmental changes. Therefore, stress proteins including HSC 70 may have important roles for the oyster under normal and stress conditions. In fact, in vitro studies revealed that the cultured fish cells constitutively synthesized HSC 70 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 oyster. As a preliminary to resolve the role of stress protein in the oysters, the author decided to clone the HSC 70-related cDNA, referred to here as Japanese oyster HSC 71 cDNA. Nucleotide sequence and expression analyses revealed that the isolated clone represents a heat shock cognate 70 cDNA.

## MATERIALS AND METHODS

### *Materials*

Live cultured Japanese oysters *Crassostrea gigas* were purchased from a culture farm in Matoya bay, Mie Prefecture. They were reared in the artificial sea water at 12℃, the water temperature in Matoya bay.

*Preparation of RNA*

Total RNA from 1 g of oyster gill 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 Oligotex-dT 30 Super (Takara).

*RT-PCR Cloning*

To obtain a partial sequence of oyster HSC 71 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 antisense primer, where R=A+G, S=G+C, Y= C+T. These correspond to the amino acid sequences, IDLGTTYS and DEAVAYGA, respectively, which are identical in Japanese flounder HSC 71,[6] human HSC 71,[7] (shown in Fig.1), bovine HSC 71,[8] and rat HSC 71.[9] Single-stranded cDNA was synthesized from 1 μg of total RNA 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 $MgCl_2$, 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 Tth DNA polymerase (Toyobo), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 mM each dNTP, and 0.2 μM each primer. The PCR condition were 30 s at 95℃, 1 min at 56℃, and 2 min at 72℃ for 25 cycles (Astec, Program Temp Control System PC-700). The resultant products of the expected size, approximately 1100 bp, were subcloned in pBluescript II KS-(Stratagene). DNA sequencing analysis revealed that there was a cDNA fragments of HSC 71-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 oyster 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℃ in a solution comprising 5 x SSC (1 x 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% polyvi-

nylpyrrolidone 100, 0.6% BSA, and 32 P labeled probe prepared with a Megaprime DNA labelling system (Amersham International PLC). After hybridization, the filters were washed four times with 2 x SSC at room temperature and three times with 2 x SSC, 0.5% SDS at 65℃ 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 dideoxynucletides and a model 373 A automated sequencer (Applied Biosystems Inc.). The sequences reported in this paper were determined on both strands.

*Northern Blot Analysis*

Oysters were acclimated at 12℃ for more than 48 h. After the acclimatization, oysters were corrected at 0, 24, 48 h at 12℃ (Fig.3 ; Control). Oysters were heat-shocked at 20, 30, or 35℃ for 0.5, 1, 2, 4, and 10 h (Fig.3 ; Heat shock). Total RNA was isolated from gill as described above. Five micrograms of total RNA was subjected to Northern blot analysis. The probe and the condition of hybridization and washing were the same as those of cDNA library screening.

*Staining16S rRNA*

As an internal control, 16 S rRNA of oyster tissues in agarose gel was stained by ethidium bromide.

## RESULTS AND DISCUSSION

*Cloning and Sequence of the Full-length cDNA for HSC71*

An RT-PCR strategy for cloning a partial cDNA of Japanese oyster HSC 71 successfully yielded a cDNA fragment of approximately 1100 bp. DNA sequencing analysis revealed that the fragment was homologous to Japanese flounder HSC 71 gene[6] (data not shown). In order to obtain a full length cDNA of oyster HSC 71, a oyster cDNA library was screened with the PCR-derived cDNA fragments as a probe. By screening of $10^5$ independent recombinant phages, ten 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 oyster HSC 71 consists of 2233 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 1977 nucleotides and corresponds 659 amino acids with a predicted mass of about 71 kDa.

```
-32                                                    GGGGAAAGGGGAAGAAATAGAACATCAGCAAC   -1

  1 M S K P A Q Q A I G I D L G T T Y S C V G V F Q H G K V E I   30
  1 ATGAGCAAGCCAGCACAGCAAGCCATTGGAATAGATCTTGGTACCACATATTCCTGTGTTGGAGTTTTCCAGCATGGGAAGGTGGAAATC   90

 31 I A N D Q G N R T T P S Y V A F T D T E R L V G D A A K N Q   60
 91 ATCGCCAACGATCAGGGTAACCGAACCACCCCCAGTTATGTAGCGTTCACAGACACAGAAAGACTGGTCGGCGACGCAGCCAAAAACCAA  180

 61 V A M N P N N T I F D A K R L I G R K F N D A S V Q S D M K   90
181 GTCGCCATGAACCCCAACAACACAATTTTTGATGCCAAGCGTCTGATCGGCAGAAAATTCAACGATGCTTCAGTACAATCCGACATGAAA  270

 91 H W P F T V I N Q A S K P M I K V E Y K G E E K T F S A E E  120
271 CATTGGCCATTCACAGTGATCAATCAAGCAAGTAAACCCATGATCAAAGTCGAGTACAAAGGGGAAGAAAAGACCTTCTCTGCTGAGGAA  360

121 V S S M V L N K M K E T A E A Y L G K T I N N A V V T V P A  150
361 GTCTCATCCATGGTCCTCAATAAAATGAAGGAAACTGCAGAAGCATATCTTGGCAAGACAATTAACAATGCCGTCGTCACAGTCCCAGCT  450

151 Y F N D S Q R Q A T K D A G T I S G L N V L R I I N E P T A  180
451 TATTTCAATGATTCCCAGCGACAGGCTACCAAGGACGCTGGTACTATCTCAGGATTAAATGTACTACGTATCATCAACGAGCCAACAGCA  540

181 A A I A Y G L D K K V G N Q S Q G E R N V L I F D L G G G T  210
541 GCCGCCATTGCTTACGGTCTTGACAAGAAAGTTGGCAACCAATCGCAAGGTGAGCGCAACGTTCTGATCTTTGACTTGGGAGGAGGAACC  630

211 F D V S I L T I E D G I F E V K S T S G D T H L G G E D F D  240
631 TTTGATGTCTCAATTCTGACCATTGAGGACGGAATCTTTGAGGTGAAATCCACATCTGGAGACACTCACTTGGGTGGTGAAGACTTTGAC  720

241 N R M V N H F I Q E F K R K H K K D I S E N K R A V R R L R  270
721 AATAGAATGGTGAACCACTTTATTCAGGAATTCAAGCGCAAACACAAGAAGGACATTTCTGAAAACAAGCGAGCTGTCCGACGTTTGAGG  810

271 T A C E R A K R T L S S S S Q A S I E I D S L F E G I D F Y  300
811 ACAGCCTGTGAAAGGGCAAAGAGGACCCTGTCCTCAAGCTCTCAGGCCAGCATTGAGATTGACTCTCTGTTCGAGGGTATCGATTTCTAC  900

301 T S I T R A R F E E L N A D L F R G T M E P V E K A L R D A  330
901 ACAAGTATCACCAGGGCTAGGTTTGAGGAGCTGAATGCCGATTTGTTCCGAGGCACCATGGAACCCGTGGAGAAGGCTCTGAGAGATGCC  990

331 K L D K A Q I H D I V L V G G S T R I P K I Q K L L Q D F F  360
991 AAGCTGGACAAGGCCCAGATCCACGACATCGTCCTGGTCGGAGGATCCACACGTATCCCAAAGATTCAGAAACTACTTCAGGACTTCTTC 1080

361 N G K E L N K S I N P D E A V A Y G A A V Q A A I L S G D K  390
1081 AACGGCAAGGAACTGAACAAATCCATCAACCCTGATGAGGCTGTTGCTTATGGAGCAGCTGTCCAGGCCGCCATTCTGTCTGGAGACAAA 1170

391 S E E V Q D L L L L D V T P L S L G I E T A G G V M T N L I  420
1171 TCCGAGGAGGTACAGGACTTGCTCCTGTTGGACGTCACCCCCCTGTCCCTGGGTATTGAAACAGCCGGAGGAGTGATGACCAATCTCATC 1260

421 K R N T T I P T K Q T Q T F T T Y S D N Q P G V L I Q V Y E  450
1261 AAGAGAAACACCACCATTCCAACCAAACAGACGCAGACCTTCACCACATACTCCGACAACCAACCAGGTGTGTTGATTCAGGTGTACGAG 1350

451 G E R A M T K D N N L L G K F E L T G I P P A P R G V P Q I  480
1351 GGAGAGCGAGCCATGACCAAGGACAACAACCTACTCGGAAAGTTCGAGCTGACTGGAATTCCCCCCGCACCCAGGGGTGTGCCCCAGATT 1440

481 E V T F D I D A N G I L N V S A V D K S T G K E N K I T I T  510
1441 GAGGTCACATTTGACATTGATGCCAACGGTATCCTGAATGTGTCAGCTGTCGACAAGAGCACAGGAAAGGAGAACAAAATCACCATTACC 1530

511 N D K G R L S K D E I D R M V N E A E K Y K Q E D E K Q R E  540
1531 AATGACAAAGGTCGCCTCAGCAAGGATGAAATTGACCGTATGGTCAATGAAGCTGAGAAATACAAACAGGAAGACGAGAAGCAGCGTGAG 1620

541 R I A A K N G L E S Y A F N M K S T V D D E K L K D K I S E  570
1621 AGGATCGCAGCCAAGAACGGGTTGGAAAGCTACGCCTTCAACATGAAATCCACCGTCGATGACGAGAAACTCAAGGACAAGATCAGCGAG 1710

571 G D K K T I L D K C E E I I K W M D Q N Q L A D K E E F E H  600
1711 GGCGACAAGAAGACAATCCTTGACAAGTGTGAGGAGATCATCAAGTGGATGGACCAGAACCAACTGGCCGACAAGGAGGAGTTCGAGCAC 1800

601 K Q K E L E G V C N P I I T K L Y Q A S G G A P G G G G M P G  630
1801 AAACAGAAGGAACTGGAGGGAGTCTGTAACCCCATCATCACCAAGCTGTACCAGGCCAGCGGTGGCGCCCCAGGGGGTGGCATGCCCGGA 1890

631 G M P N F G G G A P G G G A P G G G S G G G P T I E E V D   659
1891 GGAATGCCCAACTTTGGTGGTGGAGCCCCAGGAGGTGGTGCCCCCGGCGGTGGTAGCGGTGGTGGACCCACCATTGAGGAGGTCGACTAA 1980

1981 TTATCTAATACTCAATGCTGTACGTGAAAAATTGACTTGCTACAACGTGTCCTTTATTACTTATCTCAATGCTATCAAGAGAATAAAAGA 2070
2071 CTTGTTAACAATCTCTACACCATCAAAGGAGAACAAGACTAATTTAATGTGTTCTGATATACTAATCTCTTATTATCTATCGAGAGAAAA 2160
2161 TTGACTTGTTGCAATGTGTCTTTTGCTGATGTTGATTAAAAAATTGAAACTGAAAAAAAAAAAAAAAAAAAAA                 2233
```

**Fig.1** Nucleotide sequence of the cDNA encoding oyster HSC 71 and deduced amino acid sequence. The start (ATG), stop (TGA), and polyadenylation signal sequences are underlined.

Figure 2 shows the comparison of the nucleotides sequence of HSC 71 from Japanese oyster with those of other eukaryotes. Japanese oyster HSC 71 contains a EEVD (Glu-Glu-Val-Asp) peptide motif at C -terminal end which is the common feature of cytosolic HSC proteins in HSP 70 family.[1] Comparison with protein sequences in the SWISS-PROT database by FASTA indicated that Japanese oyster HSC 71 is 87.7% and 88.9% identical in primary structure to Japanese flounder HSC 71,[6] and human HSC 71,[7] respectively. Obviously the HSC 71 amino acid sequence has been highly conserved during eukaryotes.

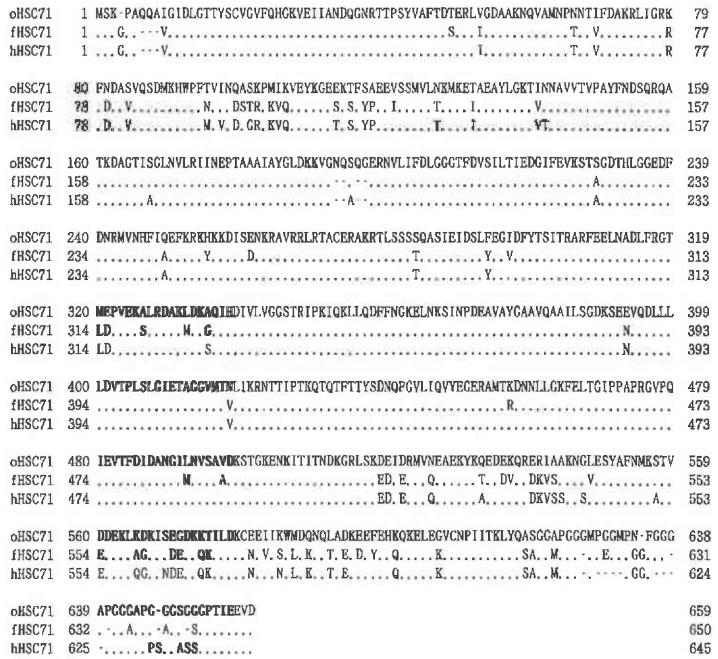

**Fig.2** Comparison of amino acid sequence of oyster (o) HSC 71 with those of other species ; Japanese flounder (f)[6] and human (h).[7] Residues identical to oyster are indicated by dots and gaps are introduced to optimize alignment by dashes.

*Constitutive Expression of Japanese Flounder HSC71 mRNA*

The expression of oyster HSC 71 gene was investigated by Northern blot analysis (Fig.3). A single hybridized signal of about 2.3 kb, which agreed well with the sizes of Japanese flounder[6] (2.2 kb), was observed in un-heat-shocked oyster gills at 12℃(Fig.3 ; Control). The oyster HSC 71 gene was also expressed constitutively under heat-shock conditions at 20, 30, or 35℃ for 0.5-10 h (Fig.3 ; Heat shock). Yamashita

reported the similar results on the platyfish HSC 70,[1] and also Yokoyama et al. on the Japanese flounder HSC 71.[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 represent a cDNA of heat shock cognate protein. The constitutive expression pattern of HSC 71 suggests the importance of HSC 71 in tissues. This fact also suggests that the HSC 71 mRNA of oyster is useful as an internal standard on Northern blot analysis.

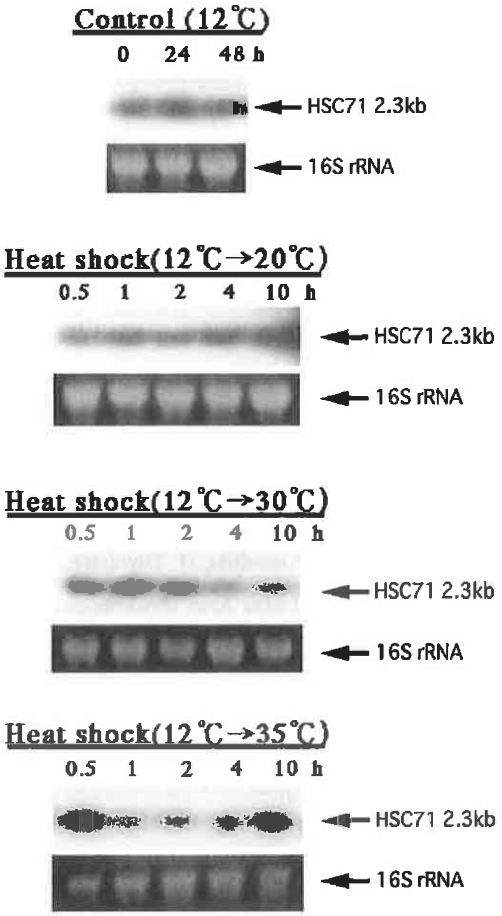


**Fig.3** Expression of HSC 71 under unstressed or stressed conditions.
Upper panel : Northern blot with HSC 71 probe.The analyses were carried out using five micrograms of total RNA from unheat-shocked (12℃ for 0, 24, and 48 h, **Control**) and heat shocked (20, 30, or 35℃ for 0.5-10 h, **Heat shock**) oyster gills.
Lower panel : 16 S rRNA of oyster gills in agarose gel was stained by ethidium bromide.

## Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

## REFERENCES

1) M. Yamashita : Genes associated with stress response, in "Fish DNA" (ed. by T. Aoki, F. Takashima, and T. Hirano), Koseisya Koseikaku, Tokyo, 1997, pp.219-243 (in Japanese).

2) K. B. Palter, M. Watanabe, L. Stinson, A. P. Mahowald, and E. A. Craig : Expression and localization of *Drosophila melanogaster* hsp 70 cognate proteins. *Mol. Cell. Biol.*, 6, 1187-1203 (1986).

3) E. A. Craig and K. Jacobsen : Mutations in cognate genes of *Saccharomyces cerevisiae* hsp 70 result in reduced growth rates at low temperatures. *Mol. Cell. Biol.*, 5, 3517-3524 (1985).

4) C. R. Brown, R. L. Martin, W. J. Hansen, R. P. Beckmann, and W. J. Welch : The constitutive and stress inducible forms of hsp 70 exhibit functional similarities and interact with one another in an ATP-dependent fashion. *J. Cell Biol.*, 120, 1101-1112 (1993).

5) T. Maniatis, E. F. Fritsch, and J. Sambrook : Molecular Cloning : A Laboratory Manual, 2 nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

6) Y. Yokoyama, H. Hashimoto, S. Kubota, M. Kinoshita, H. Toyohara, M. Sakaguchi, and M. Kanamori : Complementary DNA cloning of HSC 71, a 71 kDa heat shock cognate protein, in Japanese flounder *Paralichtys olivaceus*. *Fisheries Sci.*, 64, 388-392 (1998).

7) B. P. Dworniczak and M. E. Mirault : Structure and expression of a human gene coding a 71 kd heat shock ' cognate' protein. *Nuc. Acid Res.*, 15, 5181-5197 (1987).

8) C. D. Flaherty and D. Mckay : Nucleotide sequence of the cDNA of a bovine 70 kilodalton heat shock cognate protein. *Nuc. Acid Res.*, 18, 5569 (1990).

9) K. O'Malley, A. Mauron, J. D. Barchas, and L. Kedes : Constitutively expressed rat mRNA encoding a 70-kilodalton heat-shock-like protein. *Mol. Cell. Biol.*, 5, 3476-3483 (1985).

10) Hunt and R. I. Morimoto : Conserved features of eukaryotic hsp 70 genes revealed by comparison with the nucleotide sequence of human hsp 70. *Proc. Natl. Acad. Sci. USA*, 82, 64556459 (1985).