

Expression of GFP Gene Driven by the Olive Flounder (*Paralichthys olivaceus*) *hsc70* Promoter in Transgenic Medaka (*Oryzias latipes*)

Jeong-Ho Lee, Jong-Hyun Kim, Jae Koo Noh, Hyun Chul Kim,
Woo-Jin Kim¹, Young-Ok Kim¹ and Kyung-Kil Kim*

Genetics and Breeding Research Center, National Fisheries Research and
Development Institute, Geoje 656-842, Korea

¹Biotechnology Research Institute, National Fisheries Research and
Development Institute, Busan 619-900, Korea

Heat shock proteins (HSPs) are a family of highly conserved proteins playing an important role in the functioning of unstressed and stressed cells. The HSP70 family, the most widely studied of the hsp, is constitutively expressed (*hsc70*) in unstressed cells and is also induced in response to stressors (*hsp70*), especially those affecting the protein machinery. The HSP/HSC70 proteins act as molecular chaperones and are crucial for protein functioning, including folding, intracellular localization, regulation, secretion, and protein degradation.

Here, we report the identification and characterization of the putative amino acid sequence deduced from one cDNA clone identified as heat shock protein 70. The alignment showed that the putative sequence is 100% identical to the heat shock protein 70 cognate (HSC 70) of olive flounder. The 5'-flanking region sequence (approximately 1 kb) ahead of the *hsc70* gene was cloned by genome walking and a putative core promoter region and transcription elements were identified. We characterized the promoter of the olive flounder *hsc70* gene by examining the ability of 5'-upstream fragments to drive expression of green fluorescent protein (GFP) in live embryos.

Key words : Olive flounder (*Paralichthys olivaceus*), *hsc70* promoter, green fluorescent protein (GFP)

Introduction

The 70 kDa HSPs constitute one of the most highly conserved protein and gene families (Boorstein and Ziegelhoffer, 1994). As molecular chaperones they play essential roles in protein metabolism and protein translocation under both stress and non-stress conditions (Hartl, 1996; Bukau and Horwich, 1998; Mayer and Bukau, 1998). Among the different classes of *hsp* genes, the most abundant hsp is HSP70 family, which

is comprised of both heat (stress) inducible and constitutive expressed (cognate) members (Lindquist and Craig, 1988). A *hsp70* expressed in non-stressed cells under physiological conditions is called heat shock cognate 70 (*hsc70*) (Craig *et al.*, 1993). Such constitutive expression of *hsc70* gene has been reported in a wide variety of non-stress cells from *Drosophila*, amphibia, fish, mouse, and human (Bensaude *et al.*, 1983; Craig *et al.*, 1983; Bienz, 1984; Dworniczak and Mirault, 1987; Arai *et al.*, 1995).

In fish, *hsp70*s have been sequenced in different species including zebrafish (*hsc70*: Graser *et al.*, 1996; Santacruz *et al.*, 1997), tilapia (*hsp70*:

*Corresponding author: k48722@momaf.go.kr

Molina *et al.*, 2000), medaka (*hsp70* and *hsc70*: Arai *et al.*, 1995), and rainbow trout (*hsp70* partial sequence: Kothary *et al.*, 1984; Zafarullah *et al.*, 1992). The trout *hsc71* and *hsp70* amino acid sequences are 80% identical, whereas the human and trout *hsc70* sequences are 94% identical (Zafarullah *et al.*, 1992). In fish, there appear to be different protein isoforms for *hsp70*, however, no variation in protein isoforms was evident for *hsc70* (White *et al.*, 1994; Norris *et al.*, 1995; Place and Hofmann, 2001).

In this study, the 5'-flanking region sequence containing core promoter region and transcription elements ahead of the *hsc70* gene were identified. Using DNA constructs containing regulatory sequences of the *hsc70* gene, germline transgenic medaka expressing the green fluorescent protein (GFP) reporter gene were generated.

Materials and Methods

Olive flounder cDNA library was constructed from 10 individual fish depending on tissue type using the Uni-ZAP XR cDNA synthesis/Gigapack cloning kit (Stratagene Cloning Systems). Single-pass sequencing of the 5'-termini of selected cDNA clones in phagemid form was performed using the ABI 3100 automatic DNA sequencer (PE Applied Biosystems) and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems).

Cloning of the olive flounder *hsc70* promoter sequences was done with the Universal Genome-Walker Kit (Clontech) according to the manufacturer's protocol. Briefly, aliquots of genomic DNA (2.5 µg) were digested to produce blunt ends with 80 units of each of the following restriction endonucleases, *DraI*; *EcoRV*; *PvuII*; and *StuI*. Digested DNA aliquots were ligated separately to the GenomeWalker adaptor. Adaptor-ligated genomic DNA fragments were subjected to a primary PCR amplification with the outer adaptor primer (AP1) and an outer, gene-specific primer (GSP1). Aliquots (1 µL) of the 50-times diluted primary PCR reactions were used in the secondary PCR amplification with the nested adaptor primer (AP2) and a nested gene-specific primer (GSP2).

During primary PCR, DNA was subjected to seven cycles (94°C, 25 s; 72°C, 3 min) and 32 cycles (94°C, 25 s; 67°C, 3 min) followed by 7 min of final extension at 72°C in a Perkin-Elmer DNA Thermal Cycler 9700 (Perkin-Elmer Cetus, Norwalk,

CT). Secondary PCR had the same parameters except amplification was allowed to proceed for 5 and 20 cycles. Advantage Tth Polymerase Mix (Clontech) was used for the PCR. Major PCR bands were isolated from 1% agarose/ethidium bromide (EtBr) gels using the GeneClean III kit (BIO 101 Inc., Vista, CA), cloned into a pGEM-T Easy vector, and subjected to the dideoxynucleotide sequencing. Complete sequences were obtained using SP6 and T7 vector primers followed by extension with synthetic nucleotides.

To construct a permanent transgenic line, the vector backbone of p*hsc*-EGFP was removed by digesting with *Afl*III. Digested DNA was adjusted to 500 ng/µL in 5 mM Tris, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 100 mM KCl and 0.1% phenol red. For transient expression, an intact circular form of plasmid DNA constructs was adjusted to 100 ng/µL. Approximately 200 µl of DNA solution was injected into the blastomere of early one-cell stage embryos with a glass micropipette. At 6~24 h post injection, fish were examined using fluorescence microscopy, and GFP-expressing fish were saved.

The microinjected embryos were examined for GFP expression at various developmental stages under a fluorescein isothiocyanate (FITC) filter on a Zeiss microscope (Germany) equipped with the Axiocam video system (Zeiss). Most observations were performed using live material because visibility was the best in living fish.

Results and Discussion

1. Tissue specific heat shock protein patterns

In order to understand the molecular compositions of the olive flounder organs, the expression profiles of the identified clones in the cDNA libraries from several tissues of adult olive flounder was analyzed. Gene annotation procedures and homology searches of the sequenced ESTs were locally done by BLASTX for amino acid similarity comparisons. One of the more interesting trends among the cDNA libraries can be found in the distribution of heat shock proteins between several libraries (Table 1). In the eye and kidney cDNA libraries, a homologue for the stress related heat shock protein 70 cognate (HSC 70) was identified and a homologue to the HSP 90 was found in the kidney, testis, stomach and

liver libraries. HSPs serve in a cellular protective role. They function to facilitate the mature structural conformation of proteins, prevent denaturation of proteins under stress conditions, and renature denatured proteins. Levels of both HSP70 and HSP90 can change (usually increasing 3~15 fold) in response to cell stress (Paul and Arrigo, 2000). Heat shock protein 70 is a prominent molecule in the regulation of resistance to stress and can, amongst other functions, act as a ligand for Toll-like receptor 4 in mouse macrophages (Yang and Poovaiah, 2000). Heat shock protein 90 (also known as HSP82) interacts with steroid hormone

receptors and regulates their activity (Paul and Arrigo, 2000).

In the olive flounder testis library, the *hsp90 β* clone, together with several other clones, is the most abundant clone. As a member of the heat shock protein (HSP) family, HSP90 is a component of the inactive and metastable hetero-oligomeric structure of steroid receptors and functions as a molecular chaperone. It has been reported in rats that *hsp90* is highly expressed in primordial germ cells and continues to be expressed in both male and female premeiotic germ cells (Oh-sako *et al.*, 1995). Thus, it is likely that *hsp90*

Table 1. Tissue specific heat shock protein patterns

Clone no.	Tissue	Putative identification	Closest species	Accession no.	E-value
KIDNEY-1-C6	Kidney	heat shock protein 90 beta	<i>Danio rerio</i>	AAC21566	3.00E-78
kidney-3-C6	Kidney	heat shock protein 90 beta	<i>Platichthys flesus</i>	CAC27523	6.00E-11
kidney-3-C7	Kidney	HSC71	<i>Oncorhynchus mykiss</i>	P08108	1.00E-85
KIDN1A12	Kidney	HSPC267	<i>Homo sapiens</i>	AAF28945	3.00E-23
brain-1-F5	Brain	HSPC156 protein	<i>Homo sapiens</i>	AAH09499	2.00E-09
brain-2-A11	Brain	homolog to HSPC233	<i>Mus musculus</i>	BAB27677	1.00E-27
BRAI02C12	Brain	HSPC059 protein	<i>Homo sapiens</i>	NP_057620	3.00E-10
eyes3-D11	Eye	HSC 70	<i>Paralichthys olivaceus</i>	AAC33859	0.00E+00
stomach3-1F07	Stomach	heat shock protein 90 beta	<i>Salmo salar</i>	AAD30275	0.00E+00
HEPA2B12	Liver	heat shock protein 90 beta	<i>Danio rerio</i>	NP_571385	1.00E-06

^aIdentity (%); ^bFrequency of the clones in the sequenced pool

```
>gi|3513540|gb|AAC33859.1|
(AF053059) heat shock protein 70 [Paralichthys olivaceus]
Length = 650
Score = 371 bits (952), Expect = e-102
Identities = 186/186 (100%), Positives = 186/186 (100%)
Frame = +3

Query: 75 MSKGPAVGIDLGGTTYSVGVFQHGKVEIIANDQGNRTTFSYVAFPTDSERLIGDAAKNQVA 254
          MSKGPAVGIDLGGTTYSVGVFQHGKVEIIANDQGNRTTFSYVAFPTDSERLIGDAAKNQVA
Sbjct: 1  MSKGPAVGIDLGGTTYSVGVFQHGKVEIIANDQGNRTTFSYVAFPTDSERLIGDAAKNQVA 60

Query: 255 MNPTNTVFDKRLIGRRFDDAVVQSDMKHWPFVINDSTRPKVQVEYKGESKSFYPPEIS 434
          MNPTNTVFDKRLIGRRFDDAVVQSDMKHWPFVINDSTRPKVQVEYKGESKSFYPPEIS
Sbjct: 61  MNPTNTVFDKRLIGRRFDDAVVQSDMKHWPFVINDSTRPKVQVEYKGESKSFYPPEIS 120

Query: 435 SMVLTKMKEIAEAYLGKTVNNAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTAAA 614
          SMVLTKMKEIAEAYLGKTVNNAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTAAA
Sbjct: 121 SMVLTKMKEIAEAYLGKTVNNAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTAAA 180

Query: 615 IAYGLD 632
          IAYGLD
Sbjct: 181 IAYGLD 186
```

Fig. 1. A local alignment of deduced amino acid sequence of clone eyes3-D11 with olive flounder heat shock protein 70 cognate (accession no. AAC33859). PSI-BLAST gives this alignment a score of 371 bits, corresponding to an E value of $1 \times e^{-102}$ and identities of 100%.

plays an important role in development of germ cells.

The putative amino acid sequence deduced from one cDNA clone was identified as the heat shock protein 70 cognate (HSC 70). The alignment showed that the putative sequence is 100% identical to the heat shock protein 70 cognate (HSC 70) of olive flounder (Fig. 1). The 70 kDa HSPs constitute one of the most highly conserved protein and gene families (Boorstein and Ziegelhoffer, 1994). As molecular chaperones they play essential roles in protein metabolism and protein translocation under both stress and non-stress conditions (Hartl, 1996; Bukau and Horwich, 1998; Mayer and Bukau, 1998). Among the different classes of hsp genes, the most abundant hsp is hsp70 family, which is comprised of both heat (stress) inducible and constitutive expressed (cognate) members (Lindquist and Craig, 1988). A *hsp70* expressed in non-stressed cells under physiological conditions is called heat shock cognate 70 (*hsc70*) (Craig *et al.*, 1993). Such constitutive expression of *hsc70* gene has been reported in a wide variety of non-stressed cells from *Drosophila*, amphibia, fish, mouse, and human (Bensaude *et al.*, 1983; Craig *et al.*, 1983; Bienz, 1984; Dworniczak and Mirault, 1987; Arai *et al.*, 1995).

2. Cloning of the 5'-flanking regions of the olive flounder *hsc70* gene

To understand the regulatory networks responsible for *hsc70* gene expression in fish, one solution would be to create a transgenic fish line in which green fluorescent protein (GFP) is expressed. Following this strategy, the *hsc70* promoter region was isolated based on olive flounder EST sequences and generated germline transgenic fish expressing the GFP reporter gene under the control of this promoter region. Here this study presents a detailed characterization of transgene expression in developing fish embryo. This analysis showed that the 5'-flanking sequences of the *hsc70* promoter gene harbor all the necessary elements for specifically directing GFP gene expression. In addition, the adult transgenic fish may provide a convenient source of GFP-labeled cells or organs for in vitro functional analysis, and the large number of transgenic progeny (embryos) may also be used as an expression-based mutagenesis screen where disruption of GFP expression patterns can be observed in embryos.

To isolate the 5'-flanking regions of the *hsc70*

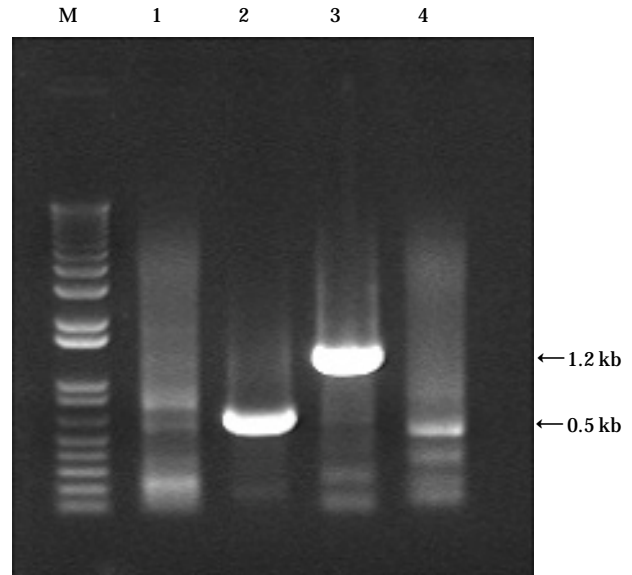


Fig. 2. Amplification of the 5'-flanking regions of the *hsc70* genes by PCR-based genomic walking strategy. Genomic DNA (2.5 μ g) were digested to produce blunt ends with restriction endonucleases, *EcoRV* (lane 1); *DraI* (lane 2); *StuI* (lane 3); and *PvuII* (lane 4). Digested DNA aliquots were ligated separately to the GenomeWalker adaptor. Lane M, 1-kb ladder of DNA size markers.

genes, a PCR-based genomic walking strategy was applied using the Universal GenomeWalker Kit and gene-specific primers GSP-1B, GSP-2B and GSP-1A, GSP-2A. Two major bands, B1 (0.5 kb) and B2 (1.2 kb), were obtained from the *DraI* and *StuI* libraries (Fig. 2). PCR products were cloned into the pGEM-T-Easy vector and sequenced. Sequencing using Bigdye terminator chemistry (Applied Biosystems) and ABI 310 DNA sequencer showed the amplified DNA fragment of about 1.2 kb had the sequence similar to the sequence of the 5'-flanking regions of the *hsc70* gene (Fig. 3) Sequence analysis of clone B1 revealed a consensus TATA sequence at position -25 relative to the transcription start site. Using the Tfscan database, several hundred putative transcription factor binding sites were found in the 1.2 kb *hsc70* upstream region (Fig. 4). In the proximal region, two potential sites for heat shock transcription factor (position -49 and -287) were found. In addition, several common response elements including several binding sites for the AP-1 protein, a TATA-like sequence and a CAAT box were found.



Fig. 3. Sequence and putative response elements in the 5'-proximal region of the olive flounder *hsc70* gene. The transcription start site of *hsc70* is numbered +1 and the translation start codon (ATG) is underlined. The potential cis-regulatory elements including several binding sites for heat shock transcription factors, the TATA-like sequence and a CAAT motif are shown in bold.

3. Expression of GFP gene driven by the olive flounder *hsc70* promoter in transgenic medaka (*Oryzias latipes*)

The pfhsc-EGFP expression vector produced by coupling 1,212 bp of 5'-flanking region of the *hsc70* gene and a partial 5'-proximal coding region to an eGFP reporter gene, was examined for its promoter activity after removal of bacterial vector sequences. In a transient transgenic analysis, although the number of fluorescent cells and intensity of fluorescence varied a little among the transient transgenic fish, high-level expression

of GFP was observed ubiquitously in the embryonic body from the morula stage or blastula stage (Fig. 5-A, B) to the 9-somite stage (Fig. 5-C, D). At the 9-somite stage, some organs, such as the yolk sac, optic vesicle, notochord and somite, could be identified on the fluorescence images. As the somites developed, the fluorescence intensity began to decrease in the somites, finally disappearing from the skeletal muscle before hatching. In hatchlings, intense fluorescence was observed in the yolk sac, lens and gill, but only weak fluorescence in the brain.

Animal strains with unique genetic markers

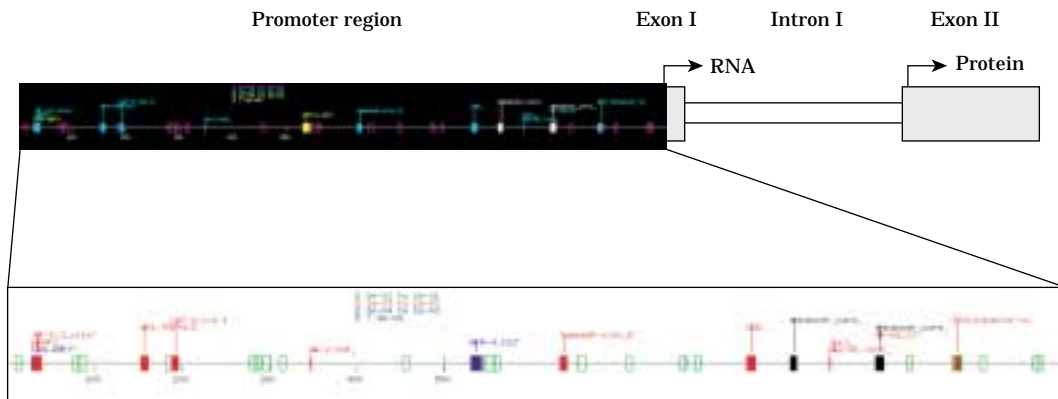


Fig. 4. Schematic diagram of several putative transcription factor binding sites in the 1.2 kb of *hsc70* promoter region.

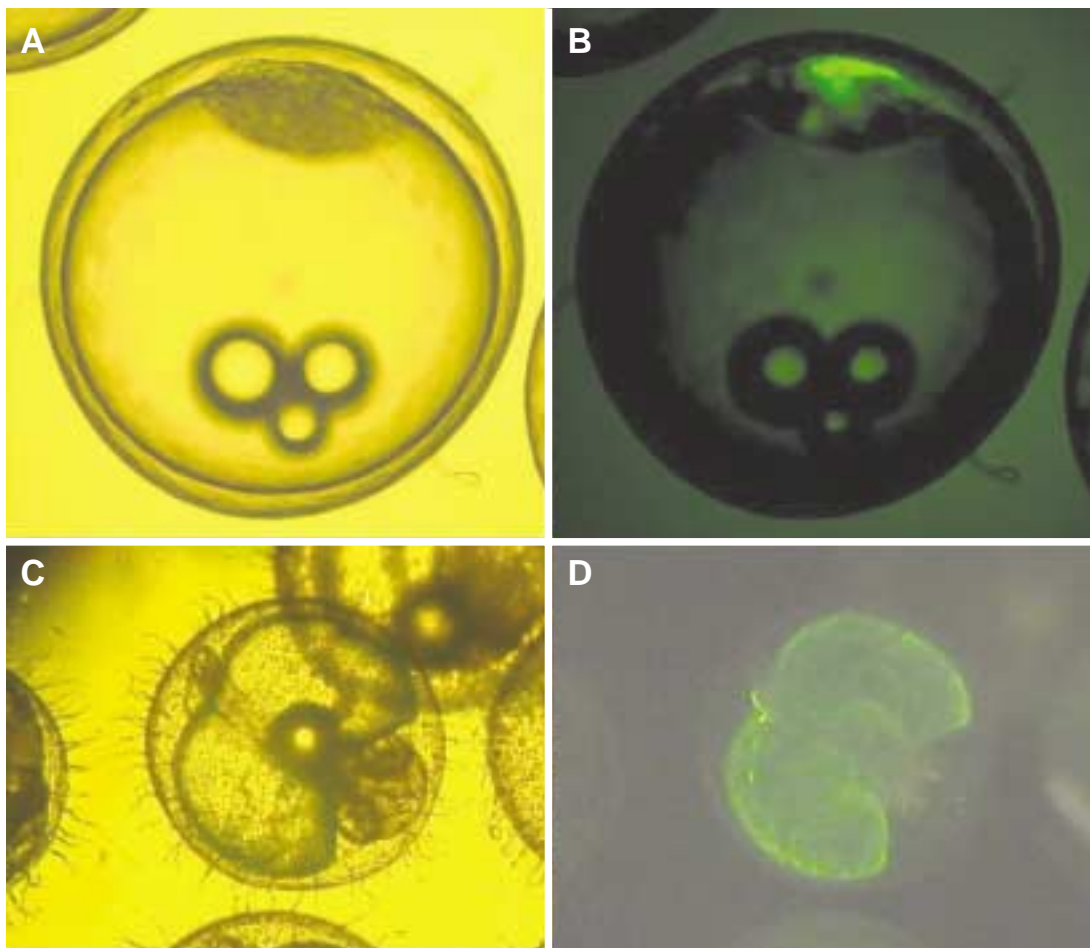


Fig. 5. Expression of GFP gene driven by the *hsc70* promoter in transgenic medaka during embryonic development. Fluorescence of green fluorescent protein (GFP) was observed ubiquitously at the blastula stage (A, B). Intense fluorescence was observed in the embryonic body at the 9-somite stage, in which organs such as the yolk sac, optic vesicle, notochord, and somites were identified in the fluorescence images (C, D).

that can be detected easily are convenient to use as donor strains for transplantation experiments.

Some natural genetic markers, such as body colors and allozymes, have been used in cellular

transplantation experiments to generate chimeras (Lin *et al.*, 1992) and also in nuclear transplantation experiments (Niwa *et al.*, 1999). However, these natural markers cannot be detected before the stages at which the embryos express these genetic markers as phenotypes. Transgenic medaka were generated in the current study using GFP as an exogenous genetic marker, which made it easy to identify donor cells or the activity of donor nuclei in living individuals from the early embryonic stages.

Acknowledgements

This work is funded by a grant from the National Fisheries Research and Development Institute (RP-2007-BT-007).

References

- Arai, A., K. Naruse, H. Mitani and A. Shima. 1995. Cloning and characterization of cDNAs for 70-kDa heat-shock proteins (Hsp70) from two fish species of the genus *Oryzias*. *Jpn. J. Genet.*, 70 : 423~433.
- Bensaude, O., C. Babinet, M. Morange and F. Jacob. 1983. Heat shock proteins, first major products of zygotic gene activity in mouse embryo. *Nature*, 305 : 331~333.
- Boorstein, W.R., T. Ziegelhoffer and E.A. Craig. 1994. Molecular evolution of the HSP70 multigene family. *J. Mol. Evol.*, 38 : 1~17.
- Bukau, B. and A.L. Horwich. 1998. The Hsp70 and Hsp60 chaperone machines. *Cell*, 92 : 351~366.
- Craig, E.A., T.D. Ingolia and L.J. Manseau. 1983. Expression of *Drosophila* heat-shock cognate genes during heat shock and development. *Dev. Biol.*, 99 : 418~426.
- Dworniczak, B. and M.E. Mirault. 1987. Structure and expression of a human gene coding for a 71 kd heat shock 'cognate' protein. *Nucleic Acids Res.*, 15 : 5181~5197.
- Graser, R.T., D. Malnar-Dragojevic and V. Vincek. 1996. Cloning and characterization of a 70-kDa heat shock cognate (hsc70) gene from the zebrafish (*Danio rerio*). *Genetica*, 98 : 273~276.
- Hartl, U.F. 1996. Molecular chaperones in cellular protein folding. *Nature*, 381 : 571~580.
- Kothary, R.K., D. Jones and E.P. Candido. 1984. 70-kDa heat shock polypeptides from rainbow trout: characterization of cDNA sequences. *Mol. Cell Biol.*, 4 : 1785~1791.
- Lin, S., W. Long, J. Chen and N. Hopkins. 1992. Production of germ-line chimeras in zebrafish by cell transplants from genetically pigmented to albino embryos. *Proc. Natl. Acad. Sci. USA*, 89 : 4519~4523.
- Lindquist, S. and E.A. Craig. 1988. The heat-shock proteins. *Annu. Rev. Genet.*, 22 : 631~677.
- Mayer, M.P. and B. Bukau. 1998. Hsp70 chaperone systems: diversity of cellular functions and mechanism of action. *Biol. Chem.*, 379 : 261~268.
- Molina, A., F. Biernar, F. Muller, A. Iyengar, P. Prunet, N. Maclean, J.A. Martial and M. Muller. 2000. Cloning and expression analysis of an inducible HSP70 gene from tilapia fish. *FEBS Lett.*, 474 : 5~10.
- Niwa, K., T. Ladygina, M. Kinoshita, K. Ozato and Y. Wakamatsu. 1999. Transplantation of blastula nuclei to non-enucleated eggs in the medaka, *Oryzias latipes*. *Develop. Growth Differ.*, 41 : 163~172.
- Norris, C.E., P.J. di Iorio, R.J. Schultz and L.E. Hightower. Variation in heat shock proteins within tropical and desert species of poeciliid fishes. *Mol. Biol. Evol.*, 12 : 1048~1062.
- Ohsako, S., D. Bunick and Y. Hayashi. 1995. Immunocytochemical observation of the 90 KD heat shock protein (HSP90): high expression in primordial and pre-meiotic germ cells of male and female rat gonads. *J. Histochem. Cytochem.*, 43 : 67~76.
- Paul, C. and A.P. Arrigo. 2000. Comparison of the protective activities generated by two survival proteins: Bcl-2 and Hsp27 in L929 murine fibroblasts exposed to menadione or staurosporine. *Exp. Gerontol.*, 35 : 757~766.
- Place, S.P. and G.E. Hofmann. 2001. Temperature interactions of the molecular chaperone Hsc70 from the eurythermal marine goby *Gillichthys mirabilis*. *J. Exp. Biol.*, 204 : 2675~2682.
- Santacruz, H., S. Vríz and N. Angelier. 1997. Molecular characterization of a heat shock cognate cDNA of zebrafish, hsc70, and developmental expression of the corresponding transcripts. *Dev. Genet.*, 21 : 223~233.
- White, C.N., L.E. Hightower and R.J. Schultz. 1994. Variation in heat-shock proteins among species of desert fishes (*Poeciliidae*, *Poeciliopsis*). *Mol. Biol. Evol.*, 11 : 106~119.
- Yang, T. and B.W. Poovaiah. 2002. A calmodulin-binding/CGCG box DNA-binding protein family involved in multiple signaling pathways in plants. *J. Biol. Chem.*, 277 : 45049~45058.
- Zafarullah, M., J. Wisniewski, N.W. Shworak, S. Schieman, S. Misra and L. Gedamu. 1992. Molecular cloning and characterization of a constitutively expressed heat-shock-cognate hsc71 gene from rainbow trout. *Eur. J. Biochem.*, 204 : 893~900.

Received : October 1, 2007

Accepted : November 22, 2007

넙치 (*Paralichthys olivaceus*) 열충격 유전자 *hsp70* 조절부위에 의한
형광단백질의 발현

이정호 · 김종현 · 노재구 · 김현철
김우진¹ · 김영옥¹ · 김경길*

국립수산과학원 육종연구센터, ¹국립수산과학원 생명공학연구소

열충격 단백질 (hsp)은 세포의 기능에 중요한 역할을 하는 보존성이 높은 단백질중의 하나이다. 이들 중 70 kDa 열충격 단백질은 외부의 자극과 관계없이 상시적으로 합성되는 HSC70 단백질과 외부의 자극에 반응하여 합성되는 HSP70 단백질이 있다. 본 연구에서는 넙치 (*Paralichthys olivaceus*)의 70 kDa 열충격 단백질에 대한 cDNA를 아미노산 서열로 변환시켜 분석함으로써 이 유전자가 상시적으로 발현하는 열충격 단백질인 HSC70에 대한 유전자임을 밝혔다. Hsp70 유전자의 발현 기작을 조사하기 위하여 단백질 발현을 조절하는 5' 인접부위를 분리하고 이들의 염기 서열을 분석함으로써 유전자 조절부위의 중요인자와 중심 부위를 동정하였다. 또한 Hsp70 유전자의 유전자 조절부위를 이용하여 형광단백질 발현벡터를 제작한 후 메다카 수정란에 미세 주입하여 배 발생 과정의 살아있는 메다카에서 발현하는 형광 단백질 (GFP)의 발현을 조사하였다.