Int. J. Indust. Entomol. Vol. 15, No. 1, 2007, pp. 75~78

[Note]

International Journal of
Industrial Entomology

Molecular Characterization of Small Heat Shock Protein(hsp20.8A) from the Silkworm, *Bombyx mori*

Jae-Sam Hwang*, Hyun-Jeong Go, Tae-Won Goo, Su-Il Seong[1], Eun-Young Yun, Mi-Young Ahn, Seong Ryul Kim, Kwan Ho Park, Iksoo Kim[2], Jae Pil Jeon[3] and Seok-Woo Kang[1]

Department of Agricultural Biology, National Institute of Agricultural Science and Technology, RDA, Suwon 441-100, South Korea

[1]Department of Life Science, College of Natural Science, University of Suwon, Hwaseong 445-743, South Korea

[2]College of Agriculture & Life science, Chonnam National University Gwangju 500-757, South Korea

[3]Korea National Institute of Health, Seoul 136-713, South Korea

(Received May 20 2007; Accepted July 30 2007)

To define the molecular mechanism of initiation and termination of diapause during the embryogenesis of silkworm, *Bombyx mori*, mRNA transcripts from diapausing eggs and diapause activated eggs were compared with differential expression using cDNA array. Among those clones, mRNA transcript from hsp 20.8A, which was expressed at a high level in diapausing eggs that had been incubated at 25°C for 30 days after oviposition, whereas, in the eggs exposed to 15°C for 30 days, 5°C for 60 days, the expression of mRNA decreased. On the other hand, the expression of mRNA during embryogenesis observed abundantly at 4 to 6 days after heat–HCl treatment and later at 9 to 10 days after just before hatching. This result was suggested for us that hsp20.8A was expressed in response to embryogenesis as well as physical stress.

Key words: cDNA array, Hsp 20.8A , Embryogenesis, *Bombyx mori*

Introduction

The silkworm, *Bombyx mori*, enters diapause at an early embryonic stage, before dermal differentiation is completed. Diapause of the silkworms continues as long as the eggs are kept at 25°C. Diapuse is terminated due to chilling at 5°C for about 3 months and when the eggs are transferred back to 25°C, embryonic development restarts. Alternatively, diapause is blocked when the eggs undergo HCl treatment at 47°C for 5 min after keeping them at 25°C for 20 hrs after oviposition.

In *B. mori* diapause eggs, several studies have been reported on the methabolism of protein, carbohydrate and lipid (Yamashita and Hasekawa, 1985; Furusawa *et al.*, 1999; Yaginuma and Yamashita, 1986) the initiation of diapause occurs accompanying with the methabolic changes such as a rapid decline in oxygen uptake (Yaginuma and Yamashita, 1990). And the accumulation of sorbitol in eggs (Horie *et al.*, 2000). Chen *et al* .(1989) noted that cyclic AMP and cyclic GMP content is changed during embryogenesis and diapause. They suggested that cGMP content is declined during diapause and is involved in early diapause. Iwasaki *et al.*,(1997) investigated the relationship between diapause and embryonic cell cycle, and Tani *et al.*, (2001, 2002) reported PIN peptide as a time-holding peptide seem to regulate the time measurement during development. Moribe *et al.*, (2001) identified *samui* as a cold inducible gene and Suzuki *et al.* (1999) suggested that *BmEts* as a novel EST family is involved in embryonic diapause.

Diapause hormone (DH), one of the neurohormones, has been identified as a major factor of inducing diapause in the resulting embryos. The expression of *DH* mRNA in the early pupal stage correlates to the incidence of diapause (Sato *et al.*,1993; Xu *et al.*, 1995). Although these findings clearly show that this hormone regulates in the induction of embryonic diapause, it is still unknown if the individual gene expression profile may regulate the stage of initiation or termination of diapause.

*To whom the correspondence addressed
Department of Agricultural Biology, National Institute of Agricultural Science and Technology, RDA, Suwon 441-100, South Korea. Tel: +82-31-290-8573; E-mail: hwangjs@rda.go.kr

Materials and Methods

Experimental animals

Silkworm hybrids between Japanese strain 123 and Chinese strain 124 were used in this study. The eggs after oviposition were preserved at 25°C for 2 months to obtain eggs in diapause stage. To obtain diapause-activated eggs, the eggs after oviposition were incubated at 25°C for 3 months and then were incubated at 5°C for 3 months, inducing the termination of diapause. After the termination of diapause, the eggs restarted their development by being incubated at 25°C.

Construction of the full-length enriched cDNA library

cDNA library from *B. mori* diapausing eggs and diapause-activated eggs was constructed by using a modification of Maruyama and Sugano's method (Maruyama and Sugano, 1994). Briefly, 100 μg of total RNA was treated with 3 units of bacterial alkaline phosphatase (TaKaRa) in 100 μl of 100 mM Tris-HCl (pH 7.5), 2 mM DTT and 80 units of RNasin (Promega) at 37°C for 60 mins. After phenol extraction and ethanol precipitation, the total RNA was treated with 100 units of tobacco acid pyrophosphatase (Wako) in 100 μl of 50 mM sodium acetate (pH 5.5), 5 mM EDTA, 10 mM 2-mercaptoethanol and 80 units of RNasin at 37°C for 60 min. The pre-treated total RNA was then ligated with 0.4 μg of 5-oligoribonucleotide (5-oligo: 5-AGC AUC GAG UCG GCCC UUG UUG GCC UAC UGG-3) using 250 units of RNA ligase (TaKaRa) in 100 μl of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.5 mM ATP, 25% PEG 8000 and 100 units of RNasin at 20°C for 3 hrs. After completing these oligo-capping reactions, mRNA was isolated using a commercial kit, QIAGEN Oligotex™. The synthesis of first-strand cDNA from the purified mRNA and cDNA amplification were performed as described by Maruyama and Sugano (1994). The amplified PCR products were then digested with *Sfi*I, and cDNAs longer than 1.3 kb were ligated into *Dra*III-digested pCNS-D2 in an orientation-defined manner. The pCNS-D2 vector contains 5 *Eco*RI - *Dra*III- *Eco*RV- *Dra* III sites at multi cloning sites, which were achieved by modifying pCNS vector (GenBank Accession no. AF 416744). The ligated cDNA was then transformed into *E. coli* Top10F' (Invitrogen) by electroporation (Gene Pulser II, BioRad). Then, cDNA sequences were determined by the dideoxy-mediated chain termination method using a 377 automatic sequencer (ABI, USA) and were analyzed by BLAST databases (http://www.ncbi.nlm.nih.gov/blastn). As a result, a total of 1,468 different cDNAs were obtained and used for cDNA expression arrays.

cDNA expression array

A total of 1,468 different cDNAs were arrayed on to Hybond-N membrane (Amersham Biosciences, Sweden) using 96-well format dot blotter (Bio-RAD, USA) after denaturation. To prepare probes, mRNAs were isolated from diapausing eggs and diapause-activated eggs. Then, ^{32}P-labeled cDNA probes were generated by reverse transcription of 0.5-1.0 μg of each poly(A)$^+$ RNA sample in the presence of [α-^{32}P]dATP. Each cDNA probe was then hybridized with the membrane at 65°C. A hybridization solution containing 50% formamide, 5x SSC, 10x Denhardt's solution (0.2% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone), 25 μg/ml sonicated salmon sperm DNA, and 50 mM sodium phosphate (pH 7.0) was used. After hybridization, membranes were washed for 30 mins with increasing stringency, from 2 × SSC and 0.1% SDS to 0.1 × SSC and 0.1% SDS. After a high-stringency wash, membranes were then exposed to X-ray film (AGFA, Germany) for 1 or 3 days at -70°C.

Northern blot analysis

Total RNAs were extracted from diapausing eggs, cold-treated eggs, HCl-treated eggs and five tissues from the 5th instar larva using SV total RNA isolation system (Promega, USA). Ten microgram of total RNA per sample was separated on 1.2% agarose/ 3% formaldehyde denaturing gel, and transferred onto a nylon membrane (Amersham). The cDNA probe was labeled with [α-^{32}P]dCTP using Prime-It II random primer labeling kit (Stratagene, USA), according to the manufacturer's instruction. Membrane was hybridized with a cDNA probe at 65°C for overnight, and analyzed by autoradiography.

Results and discussion

High-quality mRNAs were isolated from silkworm diapausing eggs and diapause-activated eggs. The mRNAs were reverse-transcribed to ^{32}P-labeled cDNAs and hybridized to a cDNA array blot which contains 1,468 Expressed Sequence Tag (EST) cDNA clones. After stringent washes and exposure to X-ray film, the expression profiles of 1,468 genes in diapausing eggs and diapause-activated eggs were obtained. By comparing duplicated hybridized blots, we identified twenty-four genes whose expression patterns were changed (Hwang *et al.*, 2005).

Using cDNA expression array, we found a number of genes that show differential expression in silkworm diapausing eggs and diapause-activated eggs(Fig. 1). We focused on the differential expression of hsp 20.8A, whose expression was significantly increased in diapaus-

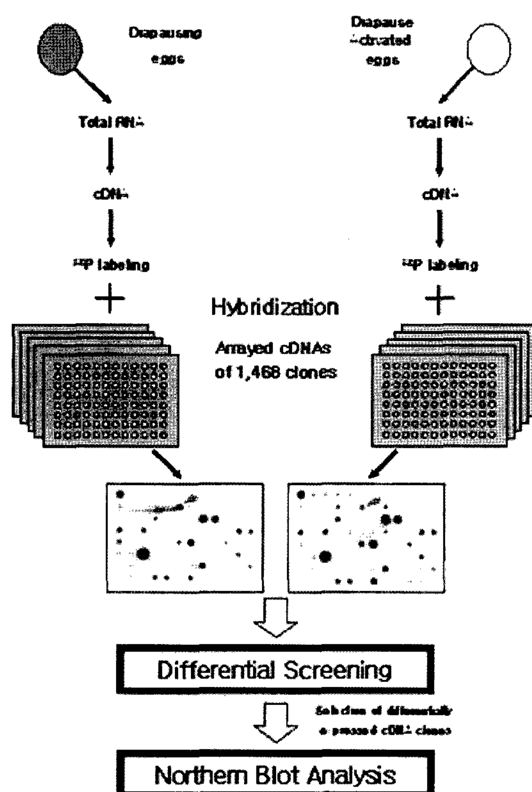

Fig. 1. The scheme of differential screening. The increased mRNA expression of *B. mori* hsp 20.8A in diapausing eggs. A total of 1,468 clones were arrayed on to Hybond-N membrane using a 96 well format dot blotter. Differential hybridization using mRNA from diapausing eggs and diapause-activated eggs as probe. The mRNA expressions of *B. mori* hsp 20.8A are indicated as an arrow.

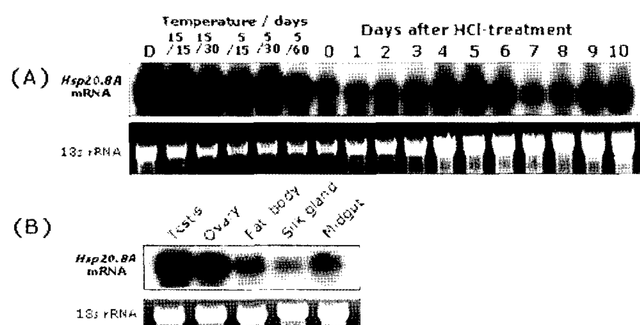

Fig. 2. Expression analyses of *B. mori* hsp 20.8A mRNA. (A) Stage-specific expression of the *B. mori* hsp 20.8A mRNA during embryogenesis. Northern blot analysis was done with total RNA samples from diapausig eggs(D), cold-treated eggs and HCl-treated eggs. As an internal control, the amount of 18S RNA was shown in the lower panel. (B) Tissue-specific expression of the *B. mori* hsp 20.8A mRNA at 5th instar larvae. Northern blot analysis was done with total RNA samples from testis, ovary, fat body, posterior silk gland and midgut. As an internal control, the amount of 18S RNA was shown in the lower panel.

ing eggs. Li *et al.*(2000) isolated and identified hsp20.8A (AF315319) in silkworm, *B. mori.* Hsp20.8A is consisted with 686 bp nucleotides and 186 amino acids (data not shown). They reported the information of molecular basis without their functional information or expression pattern. In this study, we examined hsp 20.8A mRNA expression by Northern blot analysis. As shown in Fig. 2A, hsp 20.8A mRNA was expressed at a high level in diapausing eggs that had been incubated at 25°C for 30 days after oviposition, whereas, in the eggs exposed to 15°C for 30 days, 5°C for 60 days, the expression of mRNA decreased. This result may indicate that the expression of silkworm hsp 20.8A correlate to the incidence of diapause. In other many insects, the heat shock protein family was also reported that the mRNA transcript was up-regulated during diapause. Transcripts for the two heat shock protein genes that are developmentally up-regulated in the diapause of *S. crassipalpis*, *hsp23* and *hsp70*, are not further up-regulated when diapausing pupae are subjected to heat shock or cold shock (Rinehart and Denlinger, 2000; Yocum *et al.*, 1998), but both *hsc70*, the constitutively expressed member of the *hsp70* family, and *hsp90*, a heat shock protein gene that is down-regulated during diapause, are up-regulated when diapausing flesh fly pupae are subjected to cold shock. *hsp90* is also up-regulated when the fly pupae are heat shocked (Rinehart *et al.*, 2000), but heat shock does not elicit *hsc70* upregulation in diapausing fly pupae (Rinehart and Denlinger, 2000). These results thus underscore a disynchrony of expression of the heat shock protein transcripts during diapause and in response to temperature stress during diapause. On the other hand, the expression of mRNA during early embryogenesis observed abundantly at 4 to 6 days after heat -HCl treatment and later at 9 to 10 days after just before hatching. The diapause in silkworm accompanies the developmental arrest and silkworm embryos enter blastokinesis stage at 4 to 6 days after heat -HCl treatment. In blastokinesis stage of the silkworm embryo, tissue formation and muscle development progress actively. Thus this result indicated that the expression pattern of the silkworm hsp20.8A protein correlated with an active embryogenesis.

We further examined tissue expression of hsp 20.8A mRNA in silkworm larva by Northern blot analysis (Fig. 2B). As a result, silkworm hsp 20.8A mRNA was expressed highly in testis and ovary of the 5th instar larvae.

Reference

Chen, J. H., T. Yaginuma and O.Yamashita (1989) Changes in cyclic AMP and cyclic GMP content related to embryogene-

sis and diapause of the silkworm, *Bombyx mori. J. Seric. Sci. Jpn.* **58**(1), 60-65.

Furusawa T, A. Konishi, D. Sakano, E. Kotani, Y. Sugimura, J. M. Storey and K. Storey (1999) Regulation properties of phosphofructokinase in the eggs of silkworm, *Bombyx mori. J. Insect Physiol.* **46**, 1009-1016.

Horie Y, T. Kanda and Y. Mochida (2000) Sorbitol as an arrester of embryonic development in diapausing eggs of the silkworm, *Bombyx mori. J. Insect physiol.* **46**, 1009-1016.

Hwang, J. S., H. J. Go, T. W. Goo, E. Y. Yun, K. H. Choi, S. I. Seong, S. M. Lee, B. H. Lee ,I. Kim, T. Chun and S. W. Kang (2005) The analysis of differentially expressed novel transcripts in diapausing and diapause-activated eggs of *Bombyx mori. Archives of Insect Biochemistry and Physiology* **59**, 197-201.

Iwasaki H., M. Takahashi, T. Niimi, O. Yamashita and T. Yaginuma (1997) Cloning the cDNAs encoding Bombyx homologues of Cdc2 and Cdc2-related kinase from eggs. *Insect Mol. Biol.* **6**(3), 131-141.

Li, B., Q. Y. Xia, H. Fujii and Y. Banno (2000) Isolation of small heat-shock genes (hsp20.8A) from the silkworm, *Bombyx mori. GenBank accession number* AF315319.

Maruyama K. and S. Sugano (1994) Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. *Gene* **138**, 171-174.

Moribe Y, T. Niimi, O. Yamashita and T. Yaginuma (2001) Samui, a novel cold inducible gene, encoding a protein with a BAG domain similar to silencer of death domain(SODD/ BAG-4), isolated from *Bombyx* diapause eggs. *Eur. J. Biochem.* **268**(12), 3432-42.

Rinehart J. P. and D. L. Denlinger (2000) Heat shock protein 90 is downregulated during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, but remains responsive to thermal stress. *Insect Mol. Biol.* **9**, 641-45.

Rinehart J. P., G. D. Yocum and D. L. Denlinger (2000) Developmental upregulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. Insect Biochem. Mol. Biol. **30**, 515-21.

Sato Y., M. Oguchi, N. Menjo, K. Imai, H. Saito, M. Ikeda, M. Isobe and O. Yamashita (1993) Precursor polyprotein for multiple neuropeptides secreted from the suboesophageal ganglion of the silkworm *Bombyx mori*: characterization of the cDNA encoding the diapause hormone precursor and identification of additional peptides. *Proc.Natl. Acad. Sci. U. S. A.* **90**, 3251-3255.

Suzuki M.G., T. Terada, M. Kobayashi and T. Shimada (1999) Diapause-associated transcription of *BmEts,* a gene encoding an ETS transcription factor homolog in *Bombyx mori. Insect Biochem. Mol. Biol.* **29**(4), 339-47.

Tani N, M. Isobe, S. Suwan and H. Kai (2002) PIN peptide, corresponding to fragments of egg-specific protein, interacts with EA4 to inhibit deglycosylation by PNGase F. *J. insect Biotech. Seric.* **71**, 97-102.

Tani N, T. Kamada, K. Ochiai, M. Isobe, S. Suwan and H. Kai (2001) Carbohydrate moiety of time-interval measuring enzyme regulaters time measurement through its interaction with time-holding peptide PIN. *J. Biochem.* **129**, 221-227.

Xu W. H., Y. Sato, M. Ikeda and O, Yamashita (1995) Molecular characterization of the gene encoding the precursor protein of diapause hormone and pheromone biosynthesis activating neuropeptide (DH-PBAN) of the silkworm, *Bombyx mori* and its distribution in some insects. *Biochemica et Biophysica Acta* **1261**, 83-89.

Yaginuma T and O Yamashita (1986) Malate-aspartate cycle as an effective hydrogen shuttle at the termination of diapause in the eggs of *Bombyx mori. Insect Biochem.* **16**, 677-685.

Yaginuma T and O. Yamashita (1999) Oxygen consumption in relation to sorbitol utilization at the termination of diapause in eggs of the silkworm, *Bombyx mori. J. Insect. Physiol.* **45**, 621-627.

Yamashita O. and K. Hasekawa (1985) Embryonic diapause. In comparative insect physiology, *Biochemistry and pharmacology(edited by kerkut GA and Gillbert LI) Vol. 1, Pergamone Press. Oxford,* 407-434.

Yocum G. D., K. H. Joplin and D. L. Denlinger (1998) Upregulation of a 23 kDa small heat shock protein transcript during pupal diapause in the flesh fly, *Sarcophaga crassipalpis. Insect Biochem. Mol. Biol.* **28**, 677-82.