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Construction of the Full-length cDNA Library and Selection of Diapause-Associated cDNA Clones from *Bombyx mori* Diapausing Eggs and Diapause-Activated Eggs

Jae-Sam Hwang*, Hyun-Jeong Go, Tae-Won Goo, Eun-Young Yun, Kwang-Ho Choi, Su-Il Seong[1], Sung-Kuk Kim[2], Sang-Mong Lee[3], Iksoo Kim, Nam-Soon Kim[4] and Seok-Woo Kang

Department of Agricultural Biology, National Institute of Agricultural Science and Technology, RDA, Suwon 441-100, Korea.
[1]Department of Life Science, College of Natural Science, University of Suwon, Hwaseong 445-743, Korea.
[2]Department of Life Science, College of Natural Science, Daejin University, Pocheon, 487-711, Korea.
[3]Department of Sericulture and Entomology, Miryang National University, Miryang 627-130, Korea.
[4]Genome Research Center, Korea Research Institute of Bioscience and Biotechnology, Taejon 305-333, Korea.

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As an initial step to define the molecular mechanism of diapause during embryogenesis of the silkworm, *Bombyx mori*, mRNA transcripts from diapausing eggs and diapause-activated eggs were compared by differential expression using cDNA microarray. Twenty-four individual cDNA clones were identified. Amomg them, ten genes including alcohol dehydrogenase, dead box-1, cytochrome oxidase subunit I and 18 wheeler showed increased expression in the diapause-activated eggs. The rest of fourteen genes showed increased expression in diapausing eggs.

Key words: cDNA array, Embryonic diapause, *Bombyx mori*, Differential expression

Introduction

Most insects have evolved to enter a diapause at some stage of development to survive under unfavorable environmental conditions. The environmental factors such as temperature and photoperiod determine the proper moment of a diapause program (Denlinger, 1985). Also, depends on insect species, the embryonic diapause occurs either at an early embryonic stage or much later (Chapman, 1998). The silkworm, *B. mori*, is an example of early embryonic diapause insects. The diapause of silkworm usually occurs at early embryonic stage before dermal differentiation is completed. Diapause in the silkworm 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 can be blocked by HCl treatment at 47°C for 5 min after keeping eggs at 25°C for 20 hrs after oviposition. Diapause hormone (DH), one of neurohormones, has been identified as a major factor to induce diapause in the resulting embryos (Yamashita, 1996). The expression of DH mRNA in the early pupal stage correlates with 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 for the individual gene expression profile which may regulates the stage of initiation or termination of diapause.

The genomics, the study of genes and their function, holds the potential to resolve long-standing genetic questions. A complete understanding of how genetic mechanisms control development in insects requires an accurate quantitation of gene expression. Systematic determination of the differentially expressed cDNA analysis in the specific developmental stage of insects is required to understand biological processes. As an initial step to define the molecular mechanism of diapause during embyogenesis of the silkworm, *B. mori*, we carried out differential screening using dot blot analysis.

*To whom correspondence should be addressed.
Department of Agricultural Biology, National Institute of Agricultural Science and Technology, RDA, 61 Seodun-dong Kwonsun-gu Suwon Kyounggido 441-100, 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 diapausing eggs. For obtaining 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 to induce termination of diapuse. After termination of diapause, the eggs were restarted development by incubating at 25°C.

cDNA expression array

A total of 1,468 different cDNAs were arrayed onto Hybond-N membrane (Amersham Biosciences, Sweden) using 96-well format dot blotter (Bio-RAD, USA) after denaturation. For preparing probes, mRNAs were isolated from diapausing eggs or diapause-activated eggs. Then, ^{32}P-labeled cDNA probes were generated by reverse transcription of 0.5 – 1.0 g 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, 5 × SSC, 10 × Denhardts solution (0.2% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone), 25 g/ml sonicated salmon sperm DNA, and 50 mM sodium phosphate (pH 7.0) was used. After hybridization, membranes were washed for 30 min 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.

Results and Discussion

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 Suganos method (Maruyama and Sugano, 1994). Briefly, 100 μg of total RNA was treated with 3 units of bacterial alkaline phosphatase in 100 μl of 100 mM Tris-HCl (pH 7.5), 2 mM DTT and 80 units of RNasin at 37°C for 60 min. After phenol extraction and ethanol precipitation, the total RNA was treated with 100 units of tobacco acid pyrophosphatase 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 in 100 μl of 50 mM Tris-HCl (pH7.5), 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 was achieved by modifying pCNS vector (GenBank Accession no. AF416744). The ligated cDNA was then transformed into *E. coli* Top10F by electroporation. Then, cDNA sequences were determined by the dideoxy-mediated chain termination method using a 377 automatic sequencer and were analyzed by BLAST databases (http://www.ncbi.nlm.nih.gov/BLAST/). As a result, a total of 1,468 different cDNAs was obtained and used for cDNA expression arrays.

Differentially expressed cDNAs from silkworm diapausing eggs and diapause-activated eggs

High-quality mRNAs were isolated from silkworm diapausing eggs or 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 expressions were changed (Fig. 1), as summarized in Tables 1 and 2. Only genes with expression levels that were altered more than two fold in comparisons of diapausing eggs and diapause-activated eggs are included. Ten genes showed increased expression in diapause-activated eggs. Among them, four genes had functions and other six genes were functionally unidentified (Table 1). Four functional genes were alcohol dehydrogenase, deadbox-1, cytochrome c oxidase subunit I (COI), and 18 wheeler (Bm18w). Especially, Bm18w is a Toll like molecule which acts as an adhesion molecule during various processes of morphogenesis in *Drosophila melanogaster* (Eldon *et al.*, 1994). Most of Bm18w mutants in fruit fly develop up to the third larval instar, but die prior to pupariation (Williams *et al.*, 1997). Fourteen genes showed decreased expression in diapause-activated eggs. The functions of seven out of fourteen genes are known(Table 2). Among them, embryonic abnormal visual system gene (*elav*) is a gene-specific regulator of alternative pre-mRNA processing which is important for generating an

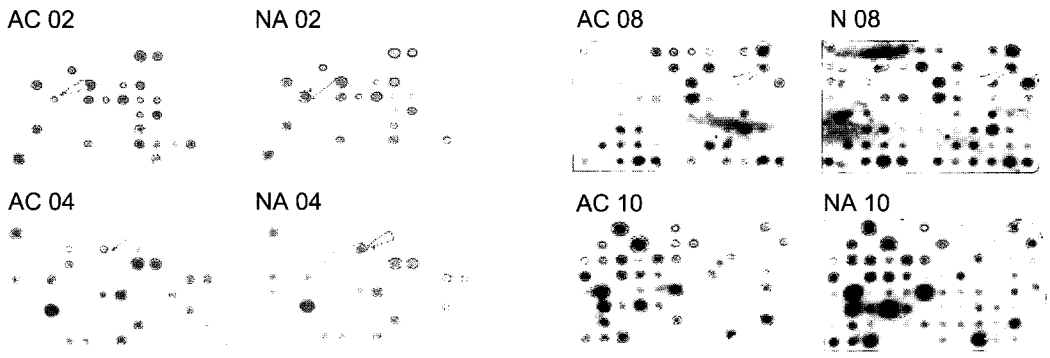

Fig. 1. A total of 1,468 unigenes was arrayed using 96-well format dot blotter in nylon membranes. Among the total of 1,468 EST clones, 24 cDNA clones were significantly expressed in diapausing eggs (NA) and diapause-activated eggs (AC). Arrows indicate some of the selected clones.

Table 1. Summary of highly expressed genes in diapause-activated eggs (AC) of silkworm

Clone no.	Gene Bank no.	Putative function	Species
0440	AF399909	alcohol dehydrogenase	*D. rerio*
0465	NM_079488	dead-box-1	*D. melanogaster*
1103	[1]NI	NI	
1931	NI	NI	
2065	NI	NI	
2086	NI	NI	
2451	NI	NI	
2776	NI	NI	
2823	AF315319	cytochrome oxides subunit I	*B. mori*
4984	AB070579	18 wheeler	*B. mori*

[1]NI: No Identity.

Table 2. Summary of highly expressed genes in diapausing eggs (NA) of silkworm

Clone no.	Gene Bank no.	Putative function	Species
0330	Y12701	ATP synthetase, gamma subunit	*D. melanogaster*
0893	AF031652	caspase-8/-10 homolog	*D. melanogaster*
1539	AF249979	ribosomal protein L8	*S. frugiperda*
1624	L08106	glycyl-tRNA synthetase	*B. mori*
1695	[1]NI	NI	
2220	NI	NI	
2522	AF288217	translationally-controlled tumor protein	*D. rerio*
2878	NI	NI	
2912	NI	NI	
2937	AF315319	heat shock protein hsp 20.8A	*B. mori*
3104	NI	NI	
4091	NI	NI	
4605	NI	NI	
4907	U28654	retinol dehydratase	*S. frugiperda*

[1]NI: No Identity.

embryonic neural system in *Drosophila* (Soller and Whiter, 2003). Caspase-8/-10 homolog (*Dcp2*) gene and translationally controlled tumor protein (*tctl*) gene were also down-regulated in diapause-activated eggs. DCP2 contains two N-terminal death effector domains fused to a caspase-like domain which is the mediator of an apoptosis (Inohara *et al.* 1997). A protein encoded by *tctl* is ubiquitously expressed and is present in evolutionarily diverse

organisms. Recently, TCT1 was reported as a regulator of translation elongation factor eEF1A in yeast (Cans *et al.*, 2003). Heat shock protein 20.8A isolated from *B. mori* by Li *et al.* (2000) but is not function. In other many insects, the heat shock protein family was reported that the mRNA transcripts were up-regulated during diapause. Thus, further studies will be necessary to clarify whether diapause-associated cDNA clones identified here are indeed responsible for maintenance and termination of diapause in *B. mori* eggs.

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