Differential Expression of the Two Cold-Inducible Genes, *Samui* and *sorbitol dehydrogenase*, in *Bombyx* Diapause Eggs Exposed to Low Temperatures

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(Received December 13, 2001; Accepted August 22, 2002)

To further understand the function of *Samui* protein which is a member of the BAG-protein family, the temporal profiles of mRNAs for the cold-inducible genes, *Samui* and *sorbitol dehydrogenase* (SDH), were examined in *Bombyx* diapause eggs exposed to 5, 0 and −10°C after incubation at 25°C for 30 days after oviposition. Incubation at 5°C activated the expression of both genes, incubation at 0°C activated only the *Samui* gene, and incubation at −10°C did not activate the expression of either gene. Although incubation at 0°C was less effective for the termination of diapause than at 5°C, from about half of the eggs that had been incubated at 0°C for 250 days, larvae hatched within 60 days after being transferred to 25°C. This changing profile in the hatching ability appears to be correlated with the amount of *Samui* mRNA. These results are consistent with a proposal from a previous study which stated that the *Samui* protein may play a role in transmitting the signal from low temperatures, such as 5 and 0°C, to a downstream cascade in a diapause egg. In addition, a possible strategy for the long-term preservation of *Bombyx* eggs was discussed.

Key words: Cold-inducible genes, *Samui*, *sorbitol dehydrogenase*, embryonic diapause, *Bombyx mori*

INTRODUCTION

Embryonic diapause of the silkworm, *Bombyx mori*, is pre-determined by the action of a neuropeptide hormone (diapause hormone) on the developing ovaries during the pupal stage (Yamashita, 1996). After oviposition and fertilization, the nuclear and cellular divisions continue until late gastrulation, when the embryonic cell cycle becomes arrested in the G₂ phase and the embryo enters diapause (Nakagaki et al., 1991).

With the initiation of diapause, sorbitol accumulates to a high concentration of about 150 mM, together with glycerol (Chino, 1958; Yaginuma and Yamashita, 1978). These polyols have been thought to function as cryoprotectants to stabilize the subcellular structure and function (Suzuki et al., 1983; Storey and Storey, 1988; Yamashita and Yaginuma, 1991), and were recently proposed to function as arresting factors of embryonic development (Horie et al., 2000). Diapause is maintained as long as the eggs are incubated at 25°C, but diapause is broken when the eggs were exposed to 5°C for about 2 months.

At the termination of diapause, sorbitol is converted into glycogen, which is eventually utilized for further embryogenesis (Yaginuma and Yamashita, 1978). This conversion is controlled by NAD-sorbitol dehydrogenase (SDH, EC1.1.1.14), which is induced at 5°C (Yaginuma and Yamashita, 1979; Yaginuma et al., 1990). The activity is regulated at the transcription level, SDH mRNA being expressed mainly within yolk cells in diapause eggs after chilling at 5°C for 40-50 days after 2 days post-oviposition (Niimi et al., 1993).

Recently, another cold-inducible gene, *Samui*, was isolated from diapause eggs (Moribe et al., 2001). The expression of *Samui* mRNA was activated after chilling the diapause eggs at 5°C for 5-6 days after incubation at 25°C for 2 days post-oviposition. *Samui* protein is shown to be a member of the BAG-protein family because it has a BAG-domain and can bind to HSP70. Consequently, *Samui* protein is speculated to play an important role in transmitting the 5°C-signal to the inside components of diapause eggs.

On the other hand, incubation at around 0°C was shown not to stimulate SDH activity in *Bombyx* diapause eggs, where a high concentration of sorbitol was maintained (Yaginuma et al., 1990). However, it is not yet known whether incubation at 0°C suppresses SDH mRNA expression. One aim of this study is to answer this question.

Further, incubation at 0°C is much less effective for the termination of diapause than at 5°C, but the long-term incubation of the eggs at 0°C causes resumption of the hatching ability although at low levels (Yaginuma et al., 1990). If the hypothesis that the *Samui* protein serves in transmitting a signal from the information of low temperatures to the downstream cascades in the eggs in order to break the diapause, is acceptable, the *Samui* gene could be up-regulated even at 0°C. This expectation was tested, and

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finally investigation was done on whether the low hatching ability after incubation at 0 and −10°C was due to diapause maintenance or cold-induced damage. Based on these results, the mechanism for the long-term storage of *B. mori* eggs is discussed.

**MATERIALS AND METHODS**

**Study organism**
Diapause eggs of a hybrid race (AsaHi x ToKai) of the silkworm, *Bombyx mori*, were used in the experiments. Larvae were reared on fresh mulberry leaves or on an artificial diet (Gunma prefecture Center) at 25-26°C. Pupae were kept at 25°C. About 12 days later, female adults emerged, copulated with males, and then deposited the eggs, still at 25°C (Yamashita and Yaginuma, 1991). The eggs laid during the first 6 hours were usually pooled and then incubated at 25°C for 30 days after oviposition to maintain diapause. Thereafter, these eggs were incubated at 5, 0 or −10°C. The sampling was usually carried out by keeping the eggs at −80°C.

**Reverse transcription-polymerase chain reaction (RT-PCR) amplification**
A real-time quantitative PCR of mRNAs of *Samui* and *sorbitol dehydrogenase* was carried out using SYBR Green I double-stranded DNA binding dye chemistry on the GeneAmp 5700 Sequence Detection System (PE Biosystem) (Moribe et al., 2001). RNA was prepared from approximately 100 eggs (about 50 mg) using TRIZOL reagent (Gibco BRL). Each 1 μg RNA sample and Superscript II (Gibco BRL) were used for the synthesis of the first strand cDNA. One fortieth of the above cDNA-synthesizing mixture and two primers, 5'-707GGAGAACCCAGTTCATTGTG207-3' and an antisense of 5'-1041AACGAGCTTCCGTTAAAGTG354-3' for *Samui* (Moribe et al., 2001), or 5'-183CCGATTGTCATTGGTCACGA202-3' and an antisense of 5'-334TGGAGCCACGTTATTGCTCC-354-3' for *sorbitol dehydrogenase* (Niimi et al., 1993), were used. For normalization, mRNA levels for *Samui* and *sorbitol dehydrogenase* were divided by the *Bombyx* ribosomal protein 49 (rp49) mRNA level that had been quantified in the same manner (Moribe et al., 2001).

**RNA isolation and Northern hybridization**
RNA was prepared from approximately 350 eggs (about 0.2 g) using TRIZOL reagent (Gibco BRL). Each 30 μg RNA sample was denatured in formaldehyde and formamide, electrophoresed in 4.0% agarose gels that contained formamide, and then blotted onto a Hybond N+ membrane (Amershams Pharmacia Biotech). Hybridization was performed in the presence of 50% formamide at 50°C, according to the usual procedures. *Samui* cDNA was radiolabeled and used as a hybridization probe (Moribe et al., 2001).

**Hatching ability**
The eggs that were incubated at 5 or 0°C were directly transferred to 25°C in order to monitor further embryonic development, whereas the eggs incubated at −10°C were exposed to 0 and 10°C, each for one day, to avoid heat damage before being transferred to 25°C. After incubation at 25°C for 60 days, the eggs were checked for hatching. Approximately 400 eggs were used in each treatment.

**RESULTS AND DISCUSSION**

**Gene expression of *Samui* and *sorbitol dehydrogenase*, and hatching ability in *Bombyx* diapause eggs exposed to 5°C after incubation at 25°C for 30 days after oviposition**
Here, using the eggs where the diapause state was completely established by incubation at 25°C for 30 days after oviposition, the effects of different low temperatures on the expression of the *Samui* and *SDH* genes were examined. In the eggs incubated at 25°C for 2 to 30 days after oviposition, the amount of *Samui* mRNA was at a low and almost constant level (Fig. 1). When the eggs were thereafter exposed to 5°C for 20 days, the amount of *Samui* mRNA increased 4-fold higher than that in the eggs before chilling. In the case where the eggs were further chilled at 5°C for 30 to 110 days, the amount of mRNA fluctuated between 3 and 5-fold that of the eggs before chilling (Fig. 1). These results were confirmed by the results from the Northern hybridization analysis (figures not shown), and they were also consistent with the previous report which stated that the *Samui* gene is up-regulated in diapause eggs by exposure to 5°C after incubation at 25°C for 2 days after oviposition (Moribe et al., 2001).

The amount of *SDH* mRNA was at a consistently low level in the eggs chilled at 5°C for 20 days from 30 days post-oviposition and resembles the level of the eggs incubated at 25°C for 2 days after oviposition (Fig. 1). The amount of *SDH* mRNA increased linearly when the eggs were further chilled at 5°C for 50 to 100 days (Fig. 1). This changing pattern in *SDH* mRNA strongly coincides with that of *SDH* activity in diapause eggs exposed to 5°C after incubation at 25°C for 30 days after oviposition (Yaginuma et al., 1990). This pattern also confirms that *SDH* activity was controlled at the transcription level, as demonstrated in diapause eggs that were exposed to 5°C from 2 days after oviposition, when the diapause state is not yet established completely (Niimi et al., 1993).

When the eggs that had been exposed to 5°C for 40 days were transferred to 25°C in order to allow post-dia-
pause development, larvae hatched from 15% of the eggs within 60 days after the transfer (Fig. 1). Larvae hatched from up to 94% of the eggs after 70 days at 5°C and plateaued at > 95% after 80-110 days at 5°C (Fig. 1). This changing profile in the hatching ability correlates with that of SDH mRNA, suggesting that the gene expression of SDH corresponds to the termination of diapause that is promoted by 5°C (Yaginuma et al., 1990; Yaginuma and Yamashita, 1999).

Gene expression of *Samui* and sorbitol dehydrogenase, and hatching ability in Bombyx diapause eggs exposed to 0°C after incubation at 25°C for 30 days post-oviposition

In the eggs that had been incubated at 0°C for 70-90 days after 30 days post-oviposition, the amount of *Samui* mRNA was approximately 8-fold greater than that in the eggs before chilling (Fig. 2). In the eggs exposed to 0°C for 100 to 200 days, the amount of mRNA increased linearly up to 16-fold, and thereafter tended to decline (Fig. 2). This result indicates that the long-term incubation of diapause eggs at 0°C after 30 days post-oviposition can up-regulate the *Samui* gene, even though the short-term incubation at 0°C was not effective for activation (Moribe et al., 2001). The reason why 0°C-enhanced levels of *Samui* mRNA are about 4-fold higher than those enhanced by 5°C (Figs. 1 & 2) could be because mRNA-degradation activities are lower at 0°C than at 5°C. Alternatively, or in conjunction with the previous explanation, the reason could come from the temperature compensation that levels of *Samui* mRNA and protein in 0°C for *Samui* protein to have the same physiological effect at 0 and 5°C must be much higher than in 5°C (Hochachka and Somero, 1984).

In contrast, there was no evidence of up-regulation of the SDH gene in the eggs exposed to 0°C throughout the experimental period (Fig. 2). This result is consistent with our earlier report that SDH enzyme activities never occur in diapause eggs exposed to around 0°C (Yaginuma et al., 1990), indicating that the enzyme activity is regulated at the transcription level.

Although the hatching ability was very low in the eggs incubated at 0°C for the first 110 days, hatching increased to 10 and 53% of the eggs after exposure to 0°C for 140 and 250 days, respectively (Fig. 2). This changing profile seemed to be positively correlated with the latter half of the *Samui* mRNA profile (Fig. 2).

To test whether the low hatching ability resulted from cold damage at 0°C or maintenance of diapause, the following experiment was carried out. When the eggs that had been exposed to 0°C for 200, 250 or 300 days were thereafter incubated at 5°C for 60 days in order to break the diapause and then transferred back to 25°C to allow post-diapause development, larvae hatched from 83, 76 and 72% of the eggs, respectively (figures not shown). This result shows that the lowered hatching ability of the eggs exposed to 0°C results from diapause maintenance rather than cold damage, and that incubation at 0°C is not suitable for the termination of diapause.

As diapause development was promoted by further exposure to 5°C in the eggs that had been incubated at 0°C (the present study; Furusawa et al., 1982; Yamashita et al., 1988; Yamashita and Yaginuma, 1991), expression of SDH activity and mRNA in such eggs is justly expected...
to be activated by 5°C. In fact, SDH activities appeared in the eggs during incubation at 5°C from around 0°C (Furusawa et al., 1992), although the amount of SDH mRNA is not yet determined. Furthermore, in the eggs from which larvae hatched after incubation at 0°C for 140-250 days and then following transfer to 25°C, the SDH gene is thought to be expressed in the course of incubation at 25°C from 0°C, without the necessity of incubation at 5°C, because the signal from the cold for diapause termination was transmitted to the downstream cascades through the expression of the Samui gene, even at 0°C.

**Gene expression of Samui and sorbitol dehydrogenase, and hatching ability of Bombyx diapause eggs exposed to −10°C after incubation at 25°C for 30 days after oviposition**

There was no increase in the amount of Samui or SDH mRNA in the eggs chilled at −10°C throughout the experimental period (Fig. 3). There was also no marked hatching within 60 days after transfer to 25°C from −10°C, through incubations at 0 and 10°C for one day each in order to avoid the heat-shock (Fig. 3).

To examine the viability of these eggs incubated at −10°C, the following experiments were performed. When the eggs that had been exposed to −10°C for 200, 250 or 300 days, were incubated at 5°C for 60 days to break the diapause, and then followed by 25°C to allow post-diapause development, they had a hatching success of 56, 18 or 1%, respectively (figures not shown). This indicates that some portions of the eggs were still living even during incubation at −10°C for the first 300 days, and that the long-term incubation at −10°C resulted in cold damage on the eggs rather than continuous maintenance of the diapause. In the eggs from which larvae hatched within 60 days at 25°C after exposure to 5°C for 60 days from −10°C, the Samui and SDH genes are supposed to be expressed during diapause development promoted by incubation at 5°C, although both mRNA amounts were not actually examined in this study.

In summary, the present results demonstrate that incubation at 5°C activates the expression of both the Samui and SDH genes, but incubation at 0°C activates the expression of only the Samui gene. Incubation at −10°C suppresses the expression of both the Samui and SDH genes. Although larvae can hatch from the eggs that have been incubated for a long time at 0°C, they cannot hatch from the eggs incubated at −10°C. This indicates that Bombyx diapause eggs can receive the signal for the termination of diapause from 5 or 0°C but not from −10°C. The signal from 5 or 0°C is transferred to Samui protein through the expression of the Samui gene, and then the protein may further transmit the signal to a downstream cascade in a diapause egg.

To preserve Bombyx eggs in the long-term, the simple incubation of diapause eggs at 0°C was adopted because this temperature does not induce SDH activities and consequently maintains a high concentration of sorbitol in the diapause eggs (Yamashita et al., 1988; Yaginuma et al., 1990; Yamashita and Yaginuma, 1991). As described above, incubation at 0°C does not completely repress the diapause termination, yet the present results indicate that there may be temperatures between 0 and −10°C that suppress the expression of the Samui gene and simultaneously do not induce severe cold-damage on the eggs. If it is possible to utilize these low temperatures, we will be able to preserve *B. mori* eggs for more than two years, by reducing the basal metabolic activity and maintaining the stable diapause state.

**ACKNOWLEDGMENTS**

We wish to thank Prof. M. Kobayashi and Dr. M. Ikeda for their helpful discussions. This research was funded by grants from the Ministry of Education, Science, Sports and Culture of Japan (08276101, 12460027 and 13045017), Grant-in-Aid (BDP) from the Ministry of Agriculture, Forestry and Fisheries, and the Research for the Future Program from Japan Society for the Promotion of Science (JSPS-RFTF99L01203).

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