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The homeodomain protein PBX participates in JH-related suppressive regulation on the expression of major plasma protein genes in the silkworm, *Bombyx mori*

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Abstract

In the silkworm, *Bombyx mori*, major plasma proteins referred to as 30K proteins are the most abundant proteins in the hemolymph of final (fifth) instar larvae. Surgical extirpation of corpora allata, the source of a juvenile hormone (JH), causes rapid accumulation of 30K proteins in the hemolymph of fourth instar larvae. The 30K protein 6G1 (30K6G1) gene was repressed in primary cultured fat body cells treated with a JH analog (JHA), methoprene. To identify the JH response element present in the promoter region of the 30K6G1 gene, we performed transfection analyses of the 5′-deletion mutants of the 30K6G1 gene using primary cultured fat body cells, gel retardation assays and in vivo footprinting analysis. The results from those analyses revealed that a JH response element exists in the sequence between positions −147 and −140. When the promoter construct mutated at positions −143, −142, and −141 was transfected to fat body primary cultured cells, the suppression effect on the reporter gene expression caused by JHA was reduced. Gel retardation assay using specific antibody revealed that a PBX protein binds to the JH response element. Northern blot analysis revealed that the gene expression of *Bombyx* PBX is enhanced in the fat body cells by JHA treatment. These results indicate that PBX proteins are involved in the JH signaling pathway and play an important role in suppressing 30K protein gene expression in the fat body of *B. mori*.

Keywords: Gene regulation; JH response element; Plasma protein; Fat body; PBX; Silkworm; *Bombyx mori*

1. Introduction

The insect fat body is an organ that is functionally similar to the vertebrate liver (Thomson, 1975; Wyatt and Pan, 1978; Shigematsu, 1985). It serves as a storage site for nutrients, as the major organ for intermediary metabolism, and as a source for the plasma proteins (Dean et al., 1985; Keeley, 1985). In the silkworm, *Bombyx mori*, the fat body of actively feeding larvae synthesizes plasma proteins and secretes them into the hemolymph (Gamo, 1978; Tojo et al., 1980; Tomino, 1985). Major plasma proteins referred to as 30K proteins, a group of structurally related proteins with molecular weights around 30,000 (Izumi et al., 1981; Sakai et al.,1988; Mori et al., 1990), are rather specific to the fifth (final) instar larvae. While negligible amounts of 30K proteins are detectable in the hemolymph until the end of the fourth larval instar, massive accumulation of these proteins in the hemolymph takes place from the early final larval instar to the pupal stage, and it has been shown that their synthesis in the fat body is developmentally regulated at the transcription level (Mori et al., 1990). 30K proteins have a high similarity in their amino acid sequence with the microvitellogenin of *Manduca sexta*, which is a female-specific yolk protein (Wang et al., 1989, 1990).

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The onset of 30K protein synthesis is restricted to early final instar larvae in concomitance with the disappearance of the juvenile hormone (JH) from the hemolymph (Izumi et al., 1981). Surgical extirpation of corpora allata, the source of JH, rapidly causes accumulation of 30K proteins in the hemolymph of the fourth instar larvae (Izumi et al., 1984). Thus, the synthesis of 30K proteins in *B. mori* appears to be suppressed by JH. It is known that JH is a major insect hormone that regulates the post-embryonic development of insects in cooperation with ecdysone (Nijhout, 1994). Both hormones control the larval ecdysis and also play an important role in almost every aspect of the development and reproduction of most insects by regulating the expression of specific genes (Nijhout, 1994; Dhadialla and Wyatt, 1983; Miura et al., 1998). In comparison with the knowledge about the mode of ecdysone action, a little is known about the molecular mechanisms of gene regulation by JH. For the purpose of investigating the JH functions on gene expression, we focused on the regulation of 30K6G1 gene expression.

In previous study, we established the primary culture system for the larval fat body cells of *B. mori* (Kishimoto et al., 1999). The fat body cells in primary culture actively synthesize larval plasma proteins including 30K proteins. We think that the fat body primary culture system is suitable for the analyses of hormonal regulation in plasma protein syntheses, because we can neglect the influence of the inactivating enzymes such as JH esterase present in the hemolymph.

In this study, we focused our efforts on the identification of the JH response element present in the upstream sequence of 30K protein genes. We clearly showed that the JH-related suppressible regulation element is present in the upstream region of 30K protein genes and that homeodomain protein PBX recognizes the element. We also showed that JH up-regulates the gene expression of PBX in the fat body cells.

2. Materials and methods

2.1. Animals

The hybrid strain (Kinshu × Showa) of the silkworm, *B mori*, was obtained from Ueda Sanshu. Larvae were reared at 25 °C on an artificial diet (Nihon Nosan Kogyo).

2.2. Materials

Methoprene, a JH analog (JHA), was kindly supplied by Dr. S. Sakurai (University of Kanazawa). The promoterless and enhancerless luciferase reporter plasmid PGB-V was purchased from Toyo Ink. The β-galactosidase expression vector pEXP38-β-gal control DNA was obtained from Invitrogen. The substrate of luciferase assay was PicaGene Luminescence Kit (Toyo Ink). Reporter Lysis Buffer (Promega) used to lyse cells. All other chemicals were purchased from commercial sources.

2.3. Primary cultures of fat body cells

Primary cultures of fat body cells from fifth instar larvae were carried out according to Kishimoto et al. (1999). Fat bodies were scraped away from opened larvae and washed with Grace's insect medium (Invitrogen). Approximately 60 mg of fat bodies were digested with 1.2 U/ml dispase I (Roche) dissolved in the medium for 2 h at 27 °C. After dispase digestion, the fat body cells were further dissociated by pipetting the suspension with a Pasteur pipette. The cells were washed twice with TC100 insect medium (JRH Biosciences). The resultant cells were suspended in a TC100 insect medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen) containing an appropriate amount of methoprene. The suspension of cells was plated onto a 60 mm-diameter tissue culture dish (FALCON) (5×10^6 cells in a dish). The plates were maintained at 27 °C in a humidified incubator.

Methoprene was diluted at $1{:}10^4$ (vol/vol) with ethanol, and the dilution was added to the medium at various final concentrations (Dhadialla and Wyatt, 1983; Berger et al., 1992). The cells were treated with an equal volume of ethanol as a control experiment.

2.4. Electroporation and reporter gene assay

Fat body cells were prepared as described above. After dispase digestion, cells were washed three times with Grace's insect medium. About 4×10^6 cells suspended in 0.5 ml of the medium were placed in a 0.4 cm gap electroporation cuvette (Bio-Rad). Each plasmid DNA of 1 pmol was added to the cuvette. Transfection was administered at a single voltage of 90 V, a capacitance of 1075 μF. After electroporation, the cells were washed once with a TC100 insect medium, suspended in the same medium supplemented with 10% FBS, and then plated onto a 60 mm-diameter tissue culture dish (FALCON) (2×10^6 cells in a dish). The cells were incubated for 48 h at 27 °C. They were then scraped from the dish with a rubber policeman, washed twice with PBS, and resuspended in 100 μl of the Reporter Lysis Buffer. They were then incubated for 15 min at room temperature. The lysate was centrifuged at 12,000*g* for 2 min at 4 °C. One hundred microliters of a luciferase assay reagent in a PicaGene Luminescence Kit was transferred to a vial. Twenty microliters of the cell lysate was added to the reagent in the vial, and the luciferase activity was assayed immediately with a liquid scintillation counter. A luciferase assay was normalized