BIOSYNTHESES OF MAJOR PLASMA PROTEINS IN THE SILKWORM, *BOMBYX MORI*

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BIOSYNTHESES OF MAJOR PLASMA PROTEINS IN THE SILKWORM, *BOMBYX MORI*

by

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ABSTRACT

Holometabolous insects drastically change their body structures during post-embryonic development and metamorphosis. Their developmental processes are mainly controlled by two insect hormones, juvenile hormone (JH) and ecdysteroid. By contrast with the study for the mode of ecdysteroid action, little is known about the mechanism how JH exerts its function. For the purpose of investigating the JH functions, I focused on the regulatory mechanisms of expression of the major plasma protein gene in the silkworm, *Bombyx mori*.

Plasma proteins termed "SP1" and "30K proteins" are synthesized by the fat body cells of the silkworm, *B. mori*, in a sex- and stage-specific manner during the larval development. I have successfully established a primary culture of the fat body cells to investigate the regulatory mechanisms of plasma protein gene expression. The primary cultures of fat body cells contained at least two cell types: small, oval cells, and large, spherical cells. The cells adhered and migrated on the culture dish. By the seventh day of cultivation, the cells have clustered to form the fat body-like structures that were maintained for at least three months. Plasma proteins were actively synthesized in the primary cultured cells isolated from the fat body of the final instar larvae only when the cells tightly adhered to the culture dish. Immunocytochemical analysis revealed that only 10-15% of the clustered cells synthesized plasma proteins in our culture system. These results indicate that the primary culture comprises heterogeneous cells which are morphologically and functionally

distinct. The patterns of SP1 syntheses in primary cultures faithfully reproduced its sex-dependency *in vivo*.

Subsequently, I established a method for introduction of DNAs into the fat body cells to analyze the regulatory element of 30K protein gene. Chimeric genes containing the 5' sequences (-1668 to +14) of 30K protein 6G1 gene fused to the firefly luciferase gene were introduced into fat body cells using a method of electroporation. Optimum introduction of DNA was obtained at 90 V, 1075 μF, 1 pmol of DNA and 4 x 10^6 cells in 0.5 ml of serum-free Grace's insect medium as an electroporation buffer using a 0.4 cm cuvette. When the constructs were transfected into fat body cells, the luciferase gene was accurately transcribed under the control of the 30K protein 6G1 gene promoter. By contrast, the fusion gene consisting of the 5' upstream region (-1480 to +16) of *B. mori* LCP30 gene and the luciferase gene did not express in the fat body cells. These results show that the promoter constructs introduced into the fat body cells exhibit their promoter-specific expression. The analysis of 5' upstream sequence of 30K protein 6G1 gene revealed that the enhancer elements are located in the sequence between nucleotide (nt) position -176 and -48 of the 30K protein gene.

It was considered that the synthesis of 30K protein might be negatively regulated by JH *in vivo*. In this thesis, I clearly showed that JH suppressed the 30K protein synthesis by use of the primary culture system of fat body cells. Next, I analyzed the JH-responsive element of 30K protein 6G1 genes using the electroporation method. Analysis using a series of deletion mutants revealed that the JH-responsive elements of 30K protein

6G1 gene are located downstream of nt position −176, overlapping with its enhancer elements.

GENERAL INTRODUCTION

Post-embryonic development of holometabolous insects can be briefly classified into three processes; growth, molting, and metamorphosis. These processes are known to be under control of major insect hormones, ecdysteroid and juvenile hormone. Both hormones control such disparate physiological and developmental phenomena as metabolism, seasonal polymorphism, and reproductive cycles, resulting in the morphological changes. Holometabolous insects offer us an opportunity for the study of developmentally regulated gene expression.

Insect hemolymph comprises hemocytes and plasma. In contrast to vertebrate blood, insect hemocytes comprise only a small fraction of hemolymph, and so insect hemolymph is not concerned with oxygen transport (Chapman 1982). Insect plasma is also quite different from vertebrate serum and is characterized by high concentrations of free amino acids, organic acid, sugars, and protein components (Wyatt 1978; Chapman 1982). The protein components in insect hemolymph are relatively limited number as compared to those in vertebrate serum. Most of these plasma proteins are synthesized in the fat body and released into hemolymph. Many plasma proteins have been isolated from a variety of insect species and characterized for their molecular properties and physiological functions. These proteins can be mainly classified into three groups. These include vitellogenins (Engelmann 1979; Hagedorn 1979; Bownes 1986), the female-specific yolk precursor proteins, lipophorins (Chino 1985; Shapiro *et al.* 1988), the lipid-

carrying shuttle proteins, and storage proteins (Roberts and Brock 1981; Levenbook 1985), the amino acid reservoirs for the development of adult tissues. Both the quality and the quantity of the plasma proteins change during the post-embryonic development and metamorphosis (Thomson 1975; Wyatt and Pan 1978). Moreover, some of these proteins are known to exhibit sexual dimorphism in hemolymph and to be regulated by hormones. Thus, the plasma protein genes provide a suitable system for analyzing the developmental, hormonal, and sex-dependent regulation of gene expression.

Storage proteins

Storage proteins are synthesized by the insect fat body and secreted into the hemolymph during larval development. Then during the prepupal period, many are taken up and stored in the fat body for later use in the synthesis of imaginal tissues (Webb and Riddiford 1988a). The two main classes of storage proteins are arylphorins which have a very high aromatic amino acid content (approximately 20% phenylalanine and tyrosine) (Tojo *et al.* 1980; Telfer *et al.* 1983), and female-specific proteins which have a relatively high methionine content (approximately 6%) and are predominantly expressed in females (Tojo *et al.* 1980; Ryan *et al.* 1985). These storage proteins can be categorized as larval plasma proteins with native molecular weights approximately 500,000, most of which consisting of six subunits (Roberts and Brock 1981; Levenbook 1985). For example, the storage proteins purified from *Bombyx mori*, SP1 (female-specific protein) and SP2 (arylphorin-type protein), have a molecular weight of 500,000 composed of six identical

subunits with a molecular weight 80,000, respectively (Tojo *et al.* 1980). On the other hand, *Drosophila melanogaster* contains two arylphorin-type storage proteins, LSP-1 and LSP-2, respectively (Roberts *et al.* 1977). Both are hexameric proteins but LSP-1 contains three non-identical subunits termed α, β, and γ, which are encoded by single copy genes, and their chromosomal locations have been mapped on different chromosomes (Smith *et al.* 1981), while LSP-2 is composed of six identical subunits.

The arylphorin-type storage proteins have been identified in many species classified into several orders of Insecta, including Diptera (Kinnear and Thomson 1975; Thomson *et al.* 1976; Roberts *et al.* 1977; Ueno and Natori 1982; Schenkel and Scheller 1986) and Lepidoptera (Tojo *et al.* 1978, 1980; Kramer *et al.* 1980; Kumaran *et al.* 1987). In Diptera, arylphorin synthesis begins in the final larval instar (Mintzas *et al.* 1983; Tamura *et al.* 1983; Powell *et al.* 1984; Schenkel and Scheller 1986; Patrinou-Georgoulas *et al.* 1987). Synthesis then ceases at wandering although the translatable mRNA remains in large quantities for up to 2 days before declining at pupariation (Sekeris and Scheller 1977; Lepesant *et al.* 1982, 1986; Powell *et al.* 1984). By contrast, in Lepidoptera, arylphorin is present in the early larval instar as well as in the final instar (Kramer *et al.* 1980; Tojo *et al.* 1980, 1981; Locke *et al.* 1982). The level of its expression appears to be dependent on nutrient supply (Tojo *et al.* 1981; Riddiford and Hice 1985; Kumaran *et al.* 1987). In *Manduca sexta* and *B. mori*, arylphorin synthesis ceases at wandering (Caglayan and Gilbert

1987) and its translatable mRNA disappears (Riddiford and Hice 1985).

The female-specific storage proteins might be restricted to Lepidoptera. These proteins appear to be regulated somewhat differently in the various Lepidoptera. In *B. mori* it is present in both male and female larvae until the final instar when it is synthesized only in the female (Tojo *et al.* 1980; Mine *et al.* 1983; Izumi *et al.* 1988). In *M. sexta* the female-specific storage protein appears only in final instar, first in the female on day 2, and then transiently in the male at wandering (Ryan *et al.* 1985).

30K proteins of B. mori

In *B. mori* another class of plasma proteins referred to as 30K proteins exists in the larval hemolymph. 30K proteins are a group of structurally related proteins with molecular weights around 30,000, and consist of at least five components encoded by 6G1, 12G1, 19G1, 21G1, and 23G1 gene (Sakai *et al.* 1988; Mori *et al.* 1991). The composition of 30K proteins differs among the strains of the silkworm (Gamo 1978; Maki and Yamashita 1997). These proteins are rather specific to the final instar larvae. While negligible amount of 30K proteins is detectable in hemolymph until the end of fourth larval instar, massive accumulation of these proteins in hemolymph takes place at the early fifth larval instar (Gamo 1978; Izumi *et al.* 1981). They are possibly one of yolk proteins on account of the following reasons: First, large amounts of 30K proteins are detectable in mature oocytes in females (Izumi *et al.* 1981). 30K proteins are not attacked by the vitellogenin protease (Ikeda *et al.*

1990) and the egg-specific protein protease (Indrasith *et al.* 1988), so that 30K proteins remained unused until vitellogenin and egg-specific proteins have been exhausted. Each component of 30K proteins is degraded in the substrate- and time-specific manner by 30K protein proteases just before larval hatching (Zhu *et al.* 1986; Indrasith *et al.* 1987). Second, 30K proteins have a high homology in primary structure with microvitellogenin of *M. sexta*, which has been shown to be a female-specific yolk protein (Wang *et al.* 1988, 1989). Moreover, the microvitellogenin gene is similar to 30K protein genes in exon/intron composition; a short first exon and a protein-coding second exon are interspersed by a single intron of several hundred base-pairs in length (Wang *et al.* 1989). However, the physiological functions of 30K proteins have not been completely established yet, since these proteins are synthesized in larvae of both sexes and the significant amounts are left in hemolymph at the end of oogenesis.

Fat body

The fat body is an insect tissue which possesses physiological functions of both vertebrate liver and adipose tissue (Thomson 1975; Wyatt and Pan 1978; Shigematsu 1985). The morphology of the fat body tissue of insects varies widely among different insect species. Generally, this tissue is consisted of loosely aggregated and compact masses of cells in thin lobes of the highly tracheated tissue that are freely suspended in the hemolymph. Thus, the fat body has ready access to nutrients, proteins, and hormones that are transported through the hemolymph. In such an environment, the fat body regulates the

levels of intermediary metabolism and is involved in the homeostatic maintenance of hemolymph proteins, lipids, and carbohydrates (Keeley 1985). It also performs developmentally specific metabolic activities that produce, store, or release components central to the prevailing nutritional requirements or metamorphic events of the insect (Haunerland and Shirk 1995).

Fat body is normally constructed from a variety of cell types. The most abundant cell type, found in all species, is the adipocyte, which synthesizes a lot of kinds of plasma proteins, and carries out all levels of intermediary metabolism and storage (Dean *et al.* 1985; Keeley 1985). In addition, the fat body cells are specialized as mycetocytes, which contain symbiotic bacteria (Houk and Griffith 1980; Keeley 1985), and urocytes, which store uric acid (Buckner *et al.* 1985; Cochran 1985).

During metamorphosis of holometabolous insects, virtually all organs and tissues change from the larval type to the adult type (Larsen 1976). In Diptera, the adult fat body is newly differentiated tissue from stem cells, and the larval fat body is histolyzed. In contrast, studies in Lepidoptera indicate that the larval fat body is broken down into single cells that reassociate to form the adult fat body (Butterworth 1972). The larval and adult fat bodies have specific functions related to the stage of development. For example, in Diptera the larval fat body produces larval serum and the adult fat body produces yolk polypeptides (Keeley 1985). Furthermore, the distinct areas of fat body exist in many insects and possess structural and functional differences. In *D. melanogaster*, the larval fat bodies are separated into six regions along the anterior-

posterior axis. The enzymes needed to synthesize ommochromes are produced predominantly in the anterior three segments. Also, two storage proteins of *D. melanogaster*, LSP-1 and LSP-2, are expressed at similar levels in all six of the fat body regions (Sato and Roberts 1983; Jowett *et al.* 1986; Paco-Larson *et al.* 1986), and then the storage proteins are sequestered preferentially by the posterior regions of the larval fat body.

Hormonal regulation of major plasma protein genes

It is known that juvenile hormone and ecdysteroid participate in the regulation of expression of many genes, including plasma protein biosyntheses (Roberts and Brock 1981; Dhadialla and Wyatt 1983; Bosquet and Calvez 1985; Levenbook 1985; Miura *et al.* 1998; Zhou *et al.* 1998a,b). Ecdysteroid is a steroid hormone with molt-inducing activity of insects, and most commonly consists of the five molecules in insects; ecdysone, 20-hydroxyecdysone, 3-dehydroecdysone, makisterone A, and 20-deoxymakisterone A (Nijhout 1994). Ecdysone is the hormone secreted by the prothoracic glands, but is relatively inactive. In most insects, the ecdysone is transferred into the active hormone, 20-hydroxyecdysone, by an enzyme in the hemolymph, epidermal cells, and fat body (Warren and Gilbert 1986; Watson *et al.* 1989; Robinson *et al.* 1991; Nijhout 1994). Juvenile hormone (JH) is a terpenoid produced by corpora allata, and plays a role in almost every aspect of insect development and reproduction, including metamorphosis, larval and adult diapose regulation, and ovarian development (Nijhout 1994; Wyatt and Davey). Five structurally related molecular forms occur in insect; JH-0, JH-I, JH-II, JH-III, and 4-methyl JH-I

(Nijhout 1994). JH-III is the widest spread of the JH. JH-I and JH-II appear to be restricted to the Lepidoptera. JH-0 and 4-methyl JH-I have been found only in the eggs of Lepidoptera (Yamamoto *et al.* 1988; Palli *et al.* 1991; Nijhout 1994).

Effects of these insect hormones on the syntheses of storage proteins have been studied on several species. In *M. sexta*, JH had no effect on arylphorin mRNA levels (Riddiford and Hice 1985) but suppressed the synthesis of the female-specific storage protein (Riddiford and Hice 1985; Webb and Riddiford 1988b). Similarly, in another Lepidoptera, including *Spodpptera litura*, *Trichoplusia ni*, and *Galleria mellonella*, the onset of female-specific storage protein synthesis can be prevented by prior JH application (Tojo *et al.* 1985; Ray *et al.* 1987; Jones *et al.* 1988). On the other hand, infusion of a large amount of ecdysteroid suppressed female-specific storage protein synthesis as well as that of arylphorin (Webb and Riddiford 1988a; Kanost *et al.* 1990).

In contrast to the regulation of arylphorin synthesis in Lepidoptera, its synthesis in Diptera appears to be induced by ecdysteroid. In a mutant *D. melanogaster* defective of ecdysteroid synthesis, the exogenously supplied ecdysteroid triggers the synthesis of arylphorins in larvae (Jowett and Postlethwait 1981; Roberts and Brock 1981).

In *B. mori*, allatectomy rapidly causes accumulation of SP1 in the hemolymph of females and males, and the application of JH after allatectomy suppresses SP1 synthesis (Tojo *et al.* 1981; Kajiura and Yamashita 1989). On the other hand, the concentration of SP2 in the hemolymph stayed unchanged by the JH application, as observed with other lepidopteran arylphorin.

The level of ecdysteroid in hemolymph is known to exert little effect on the synthesis of both storage proteins (Tojo *et al.* 1981).

The synthesis of 30K proteins in *B. mori* appears to be suppressed by JH. The concentration of JH in the *B. mori* hemolymph is maintained above threshold until the end of the fourth larval instar, but the level severely declines at the early final larval instar and at same time the synthesis of 30K proteins occurs (Gamo 1978; Izumi *et al.* 1981; Nijhout 1994; Wyatt and Davey). Allatectomy at the onset of the fourth larval instar provokes an immediate rise in the hemolymph concentrations of the 30K proteins, and accumulation of 30K proteins is delayed about 24 hours by applying JH to the early fifth instar larvae (Izumi *et al.* 1984). By contrast, the synthesis of 30k proteins appears to be induced by the rise in the hemolymph concentrations of ecdysteroid. Ecdysteroid stimulates the synthesis of the 30K proteins in the cultured fat body of *B. mori* (Izumi 1981). This is in agreement with the observation that the microvitellogenin synthesis of *M. sexta*, a high homologous protein with 30K proteins of *B. mori*, was induced by application of ecdysteroid (Wang *et al.* 1989).

Scope of project

A central issue in developmental biology of multicellular organism is to understand the mechanisms by which the morphogenesis is regulated by the stage-, tissue-, or sex-restricted expression of specific genes in response to extracellular factors. Studying the development of insects is suitable for the purpose, since the growth of insects can be

controlled by only a few hormones, including JH and ecdysteroid. Thus, I adopted to study the biosynthesis of major plasma proteins, 30K proteins, in *B. mori* based on the following points. First, *B. mori* is the domesticated insect with well-characterized background on the basis of physiology and genetics. Second, 30K protein genes exhibit stage-dependent expression during post-embryonic development. Third, the biosyntheses of plasma proteins, including 30K proteins, in *B. mori* is known to be regulated by JH and ecdysteroid, as well as that of other species. Recently, a cell-free transcription system was developed from the nuclear extract of the larval fat body cells in *B. mori* and the system preferentially transcribed homologous plasma protein genes in a promoter specific manner (Mine *et al.* 1995). It is, in many case, difficult to reproduce the complete control mechanism of gene expression in a cell-free transcription system. Here, to investigate the regulatory mechanisms of 30K protein syntheses, I attempted to establish primary cultures from the fat bodies of *B. mori* and to introduce the 30K protein gene constructs into the cultured fat body cells.

In Section I of this thesis, I present a method for primary culture of *B. mori* fat body cells and characterize the profiles of plasma protein syntheses in the primary culture. In Section II, attempt was made to introduce the gene constructs into the primary culture cells of the fat body. I describe a series of experiment to locate the enhancer and the JH-responsive elements in the 5' upstream region of 30K protein gene.

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Section I

Biosynthesis of major plasma proteins in the primary culture of the fat body cells from *Bombyx mori*

INTRODUCTION

Plasma proteins of holometabolous insects provide a unique opportunity for studying the developmental regulation of gene expression. In the silkworm, *Bombyx mori*, two groups of proteins referred to as "storage proteins" and "30K proteins," respectively comprise major protein constituents of the larval hemolymph (Gamo 1978; Tojo *et al.* 1980; Tomino 1985). These proteins are synthesized by the fat body cells and released into the hemolymph in a time-dependent sequential fashion during post-embryonic development and metamorphosis (Gamo 1978; Tojo *et al.* 1980; Tomino, 1985). The genomic as well as mRNA sequences for these proteins have been cloned, and their structures have been elucidated (Sakurai *et al.* 1988; Fujii *et al.* 1989; Mori *et al.* 1991). Northern blot analyses of the fat body RNA have demonstrated that the biosyntheses of these proteins are regulated in a stage-, tissue-, and sex-dependent manner at the level of gene transcription in fat body cells (Izumi *et al.* 1988; Sakai *et al.* 1988; Sakurai *et al.* 1988; Fujii *et al.* 1989). Recently, an efficient cell-free transcription system was developed from the nuclear extract of the larval fat body, which preferentially transcribed a homologous storage protein gene in a promoter specific manner (Mine *et al.* 1995).

These lines of information indicate that the core promoter region of the storage protein gene is essential for tissue-specific gene transcription. However, since it is generally difficult in experiments employing a cell-free system to corroborate the functions of regulatory elements located far away from the core promoter, it remains obscure whether the gene

segments distal to the core promoter might also participate in the regulated expression of storage protein genes. As an alternative to approaches using a cell-free system, the introduction of cloned gene constructs into native or cultured fat body cells as well as the analyses of their modes of expression facilitate further studies on the functions of sequence elements responsible for the regulated expression of plasma protein genes. In the hope of establishing an *in vitro* assay system using cultured fat body cells for the transcriptional regulation of plasma protein genes, I attempted in the present study to develop a primary culture system of the fat body cells from *B. mori* larvae. In this section, I describe the procedures for the isolation and *in vitro* culture of fat body cells. In this system, the cells in the culture were viable at least for three months and faithfully reproduced their *in vivo* function, producing storage proteins in a sex-dependent fashion.

MATERIALS AND METHODS

Materials

Grace's insect medium (Grace 1962), TC100 insect medium (Ferkovich *et al.* 1994), and fetal bovine serum (FBS) were purchased from Gibco BRL. Dispase I was obtained from Boehringer Mannheim. L-[^{35}S]-Methionine and [γ-^{32}P]ATP were from American Radiolabeled Chemicals Inc. and the Hungarian Academy of Sciences, respectively. Protein A-Sepharose was from Pharmacia. ENLIGHTNING was from Dupont/NEN. Rabbit IgG preparations against *B. mori* SP1, 30K proteins, and a larval cuticle protein (LCP22) were prepared as described previously (Izumi *et al.* 1981; Mine *et al.* 1983; Nakato *et al.* 1997). Vectastain ABC reagent was purchased from Vector Laboratories.

All other chemicals were purchased from commercial sources.

Animals

The hybrid strain (*Kinshu x Showa*) of the silkworm, *Bombyx mori*, was obtained from Kanebo Silk Elegance Co. Larvae were reared at 27°C on an artificial diet (Katakura Co.)

Establishment of primary cultures from fat body cells

Fat bodies were aseptically dissected from the larvae as follows: The larva to be sacrificed was tightly ligated with thread at sites just behind the head capsule and in front of the hind legs, respectively, and the body surface was disinfected by washing with ethanol. The dorsal cuticle of the larva was cut longitudinally and pinned open on a silicone-rubber plate. Malpighian tubules and tracheae were carefully

removed. Whole fat bodies from an individual larva were scraped away from the inside of the epidermal tissues and washed twice with Grace's insect medium. Approximately 60 mg of fat bodies from four larvae were digested with 1.2 U/ml dispase I in the medium for 2 hr at 27°C with occasional gentle shaking. 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 and once with the same medium containing 10% FBS. The resultant cells were suspended in TC100 insect medium supplemented with 10% FBS and plated onto a 96-well tissue culture plate (FALCON). In general, approximately 10^5 cells were seeded in a well, and plates were maintained at 27°C in a humidified incubator. All operations described above were performed under aseptic conditions.

Immunocytochemistry

Cultured fat body cells were washed three times with phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM Na-phosphate buffer, pH 6.5), fixed for 2 hr with 4% formaldehyde in ice-cold PBS (pH 6.5), rinsed in distilled water, and blocked with 0.5% hydrogen peroxide for 5 min as described by Easton and Horwath (1994). Following instructions provided by Vector Laboratories, the cells were rinsed in 0.1 M Na-phosphate buffer (pH 7.4) and then covered for 2 hr with each 1:1000 dilution of anti-SP1, anti-30K protein, or anti-LCP22 antibody. This was followed by rinses and a 1-hr exposure to goat anti-rabbit IgG conjugated to peroxidase. The bound peroxidase was visualized by treatment with 4-chloro-1-naphthol (Izumi *et al.* 1987).

Protein synthesis in the cultured cells

To detect protein synthesis in the primary cultures, cells were harvested at intervals as indicated and washed with sterile PBS (pH 7.0). Cells from each well (10^5 cells) were incubated in 50 μl of PBS (pH 7.0) containing 30 μCi [^{35}S]-methionine for 3 hr in a 27°C humidified incubator.

Preparation of antibody-coupled protein A-Sepharose

Each 0.2 mg of anti-SP1 IgG or anti-30K protein IgG was mixed with 60 μl of a 1:1 slurry of protein A-Sepharose/Tris-buffered saline (TBS; 20 mM Tris-HCl, 150 mM NaCl, pH 7.5) and the mixture was incubated for 1.5 hr at room temperature with gentle shaking. Unadsorbed IgG was removed by washing three times with TBS containing 0.02% Tween 20. The antibody-coupled protein A-Sepharose thus prepared was stored as 1:1 slurry in TBS at 4°C.

Immunoadsorption

The immunoadsorption of plasma proteins synthesized in a primary culture was carried out according to the method of Ausubel *et al.* (1987). After incubation for protein synthesis, cells were dissolved by adding 50 μl of a 2 x lysis buffer (260 mM NaCl, 100 mM KCl, 1% Triton X-100, 20 μg/ml pepstatin, 0.4 mM phenylmethylsulfonyl fluoride and 100 mM Hepes-KOH, pH 7.0), and the lysate was centrifuged for 30 min at 10,000 x *g*. The supernatant was incubated with 20 μl of a 1:1 slurry of protein A-Sepharose/TBS for 2 hr at room temperature with gentle shaking, and the mixture was centrifuged for 1 min at 200 x *g*.

The precleared supernatant of cell-lysate was added to each 60 μl of anti-SP1 or anti-30K protein antibody-coupled protein A-Sepharose/TBS, and the mixture was incubated for 1.5 hr at room temperature with gentle agitation. The Sepharose was precipitated by centrifugation and washed four times with TBS containing 0.02% Tween 20. Proteins bound to the Sepharose were eluted three times with each 50 μl of 0.1 M acetic acid. The combined eluate was dried in vacuo and subjected to SDS gel electrophoresis and fluorography.

Electrophoresis and Fluorography

The dried eluate from the immunoadsorption Sepharose was dissolved in 100 μl of Laemmli's SDS sample buffer (Laemmli 1970) by heating for 5 min in boiling water. Electrophoresis was performed according to the method of Laemmli on 10% polyacrylamide slab gel (Laemmli 1970). After electrophoresis, proteins were stained with Coomassie blue, and radioactive proteins were visualized by fluorography using ENLIGHTNING as described by the manufacturer.

Primer Extension Analysis

Total RNA was extracted from the cultured fat body cells according to Chomczynski and Sacchi (1987). Each 4 pmol of SP1 primer 228 (5'-TATACTCCTTGGCGATCTCC) or 30K6G1 (30K protein component 6 gene; Mori *et al.* 1991) primer 174 (5'-CGGTCTCGTATTCACCAATG) was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Primer extension was carried out using 3 μg of total RNA and each 5' end-labeled primer (1 x 10^5 cpm) as described by Mine *et al.* (1995). The synthesized cDNA was

separated on 6% acrylamide-7 M urea gel, and radioactivity was detected by autoradiography.

RESULTS

Establishment of primary cultures of fat body cells

For routine primary cultures, fat bodies were dissected from four larvae of the silkworm, *Bombyx mori*. Before dissection, the larvae were tightly ligated with thread at two locations, as described in "MATERIALS AND METHODS". This procedure prevented contamination of bacteria from the gut when the larvae were pinned on the silicone-rubber plate. The fat body cells of *B. mori* are enveloped with a basal lamina (Fig. 1a) which hampers the liberation of cells. When the fat bodies were treated with trypsin, the basal lamina remained undigested, and mucous substances and lipid drops accumulated in the reaction mixture during digestion. These substances are supposed to be released from injured cells (data not shown). I then used dispase I to digest the basal lamina. This enzyme has been known to cleave the basement membrane in mammals (Matsumura *et al.* 1975a, b; Glyn and Brunt 1985). The isolated fat bodies were treated with dispase I, as detailed in "MATERIALS AND METHODS". The concentration of dispase I and the duration of the digestion period were chosen so that the basal lamina of fat body cells was completely digested to yield a fairly even suspension of cells. By this method, approximately 10^7 cells were obtained from the fat bodies of four larvae. A TC100 medium supplemented with 10% FBS without antibiotics was selected as the culture medium. Figure 1b shows the cells just after dispersion from the fat bodies.

Morphological features of primary cultures

The morphological changes of the primary culture of the fat body cells isolated from the fifth instar day-1 female larvae are shown in Fig. 1. At least two types of cells, i.e. large, spherical cells and small, oval cells, were observed in the culture. Most cells belonged to the latter category. A nucleus and a number of oil droplets were observed in both kinds of cells. The small, oval cells were loosely adhered to the surface of the dish within 4 hr after plating (Fig. 1b). On the second day of cultivation, most of the cells adhered tightly to the bottom of dish, and the adherent cells began to migrate. After five days of cultivation, the adherent cells aggregated to form cell clusters on the surface of the dish, and parts of these clusters spread to form a thin layer (Fig. 1c). After ten days of cultivation, the clustered cells appeared to regenerate the basal lamina, forming a structure resembling that of fat body tissue (Fig. 1d). On the other hand, a portion of the large, spherical cells formed cell clusters together with the small, oval cells (data not shown). The clustered cells in the primary cultures survived for at least three months and maintained their "fat body-like" structure.

To clarify whether the cultured cells synthesize plasma proteins, I stained the clustered cells on the seventh day of cultivation with anti-SP1 (one of the major plasma proteins) and anti-30K protein antibodies. As shown in Fig. 2a, b, the cultured cells synthesized SP1 and 30K proteins. However, staining of cells with anti-LCP22 (a larval cuticle protein; Nakato *et al.* 1997) antibody showed negligible peroxidase activity in the control (Fig. 2c). Interestingly, only subsets

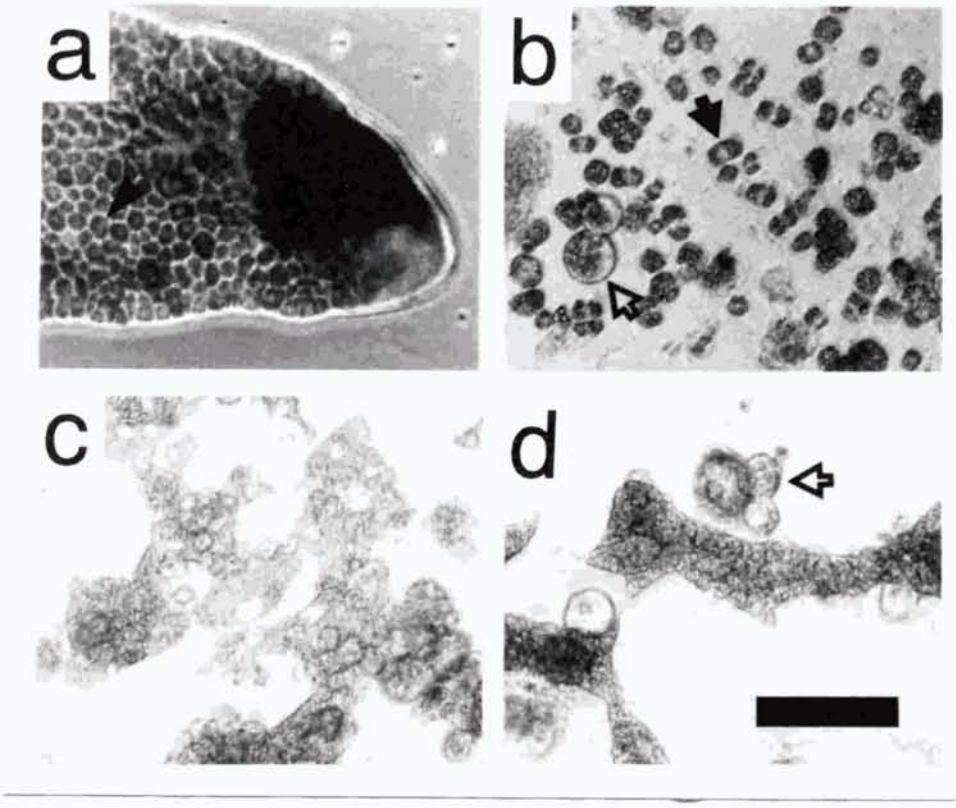


Fig. 1a-d. The morphology of primary cultures of fat bodies. The fat body cells dissected from female larvae on the fifth instar day-1 were plated onto a 96-well tissue culture plate as described in "MATERIALS AND METHODS." The fat body cells (*arrowhead*) before dispersion are enveloped with a basal lamina (a). The primary culture cells just after seeding (b), on the fifth day (c), and on the tenth day (d) of cultivation were photographed under phase contrast microscopy. The primary culture comprised two heterogeneous cell types: large, spherical cells (*open arrow*), and small, oval cells (*closed arrow*). Most cells belonged to the latter type. Both types of cells contained numerous lipid particles. Bar = 100 μm.

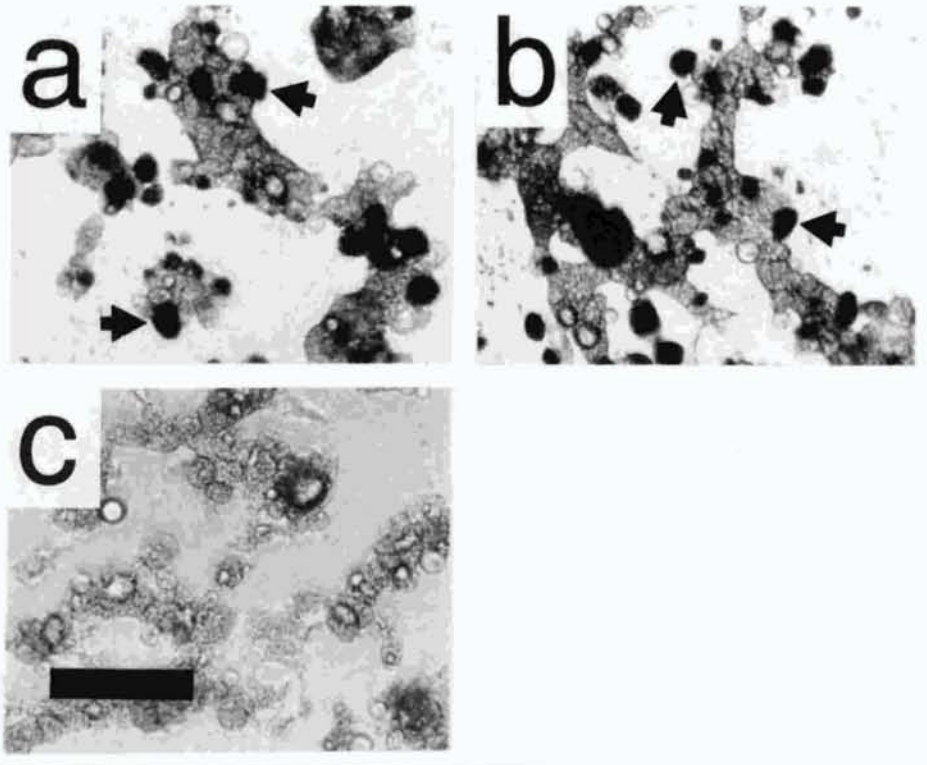


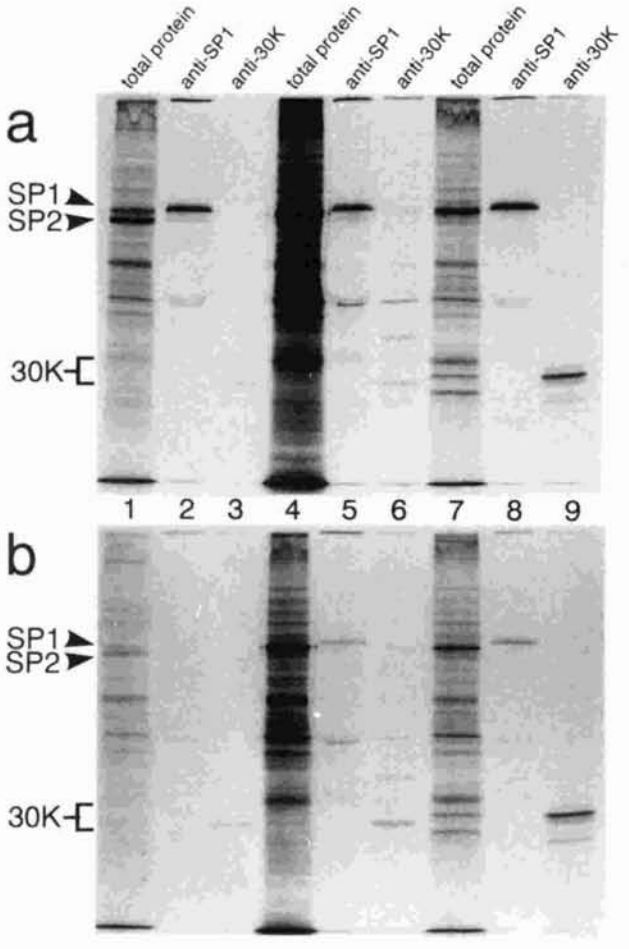
Fig. 2a-c. Immunocytochemistry of *B. mori* fat body primary cultures. The fat body cells were dissected from female larvae on the fifth instar day-1 and seeded onto a 96-well tissue culture plate. On the seventh day of cultivation, the clustered cells were stained with anti-SP1 (a), anti-30K protein (b), or anti-LCP22 (a larval cuticle protein) antibody (c). The arrows indicate the stained cells. Bar = 100 μm.

(10-15%) of clustered cells were immunoreactive in our culture system, indicating that the cells are functionally heterogeneous.

Protein synthesis of fat body cells in primary culture

Fat body cells of the final (fifth) instar larvae synthesize massive quantities of plasma proteins, releasing them into the hemolymph (Gamo 1978; Tojo *et al.* 1980; Tomino 1985). In *B. mori*, storage proteins occur in two forms, which are termed "SP1" and "SP2," respectively. SP1 is a methionine-rich storage protein exhibiting female-specific expression in the fifth instar larvae, while SP2 is an arylphorin containing a high proportion of phenylalanine and tyrosine (Tojo *et al.* 1980). "30K proteins," a group of structurally related proteins, are the most abundant proteins in the hemolymph at late-larval to early-pupal stages (Gamo 1978; Izumi *et al.* 1981), and their synthesis in the fat body is greatly stimulated by the disappearance of the juvenile hormone from the hemolymph (Gamo 1978; Izumi *et al.* 1984). In the present experiments, SP1 and 30K proteins were selected as marker proteins, and their syntheses in the cultured cells were studied.

Fat bodies dissected from male and female larvae on the fifth larval instar day-1 were dispersed with dispase treatment, and cells were cultured *in vitro*. As shown in Fig. 3a, immediately after dispersion from the female fat bodies, cells synthesized mainly SP1 and SP2 (lane 1). The rate of total protein synthesis in the cells markedly rose after cultivation for 24 hr and then progressively declined during cultivation for 7 days (lanes 1, 4, and 7). By contrast, SP1 was synthesized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1 2 3 4 5 6 7 8 9
b
SP1
SP2
30K

Fig. 3a, b. The developmental changes of total and plasma protein syntheses in primary cultures from female and male larvae on the fifth instar day-1. The fat body cells dissected from female (a) and male (b) larvae on the fifth instar day-1 were plated onto a 96-well tissue culture plate as described in "MATERIALS AND METHODS." Cells were harvested on the day of seeding (lanes 1, 2, and 3), on the first day of cultivation (lanes 4, 5, and 6), and on the seventh day of cultivation (lanes 7, 8, and 9). Each cell preparation was incubated with 50 μl of PBS containing 30 μCi [^{35}S]-methionine for 3 hr at 27°C. The cells were dissolved in each 50 μl of a 2 x lysis buffer. After centrifugation, the supernatant was treated with anti-SP1 (lanes 2, 5, and 8) or anti-30K proteins (lanes 3, 6, and 9) antibody, as described in "MATERIALS AND METHODS," and immunoadsorbed proteins were electrophoresed and fluorographed. Lanes 1, 4, and 7 represent total proteins synthesized in each preparation.

at nearly a constant rate throughout cultivation (lanes 2, 5, and 8).

To study SP1 biosynthesis at the level of mRNA, I measured the developmental change in the amount of SP1 mRNA in the cultured cells from female larvae on the fifth instar day-1 by means of primer extension. Consistent with patterns of protein synthesis, the amount of SP1 mRNA was also detectable at roughly the same level during cultivation (Fig. 4a). The result indicates that the biosynthesis of SP1 is regulated at the level of gene transcription and eliminates the possibility that the pattern of SP1 synthesis reflects the saturation of immunoadsorption.

The profile of protein synthesis in the cultivated fat body cells from male larvae was similar to that observed in the female cells, except that SP1 synthesis was severely repressed in the male cells (Fig. 3b). This clearly reflects the *in vivo* situation of female specificity of SP1 synthesis (Izumi *et al.* 1988). Although SP1 synthesis slightly resumed after cultivation for 24 hr, the rate of its synthesis was much lower than that observed with female cells throughout cultivation (lanes 2, 5, and 8).

The synthesis of 30K proteins was only barely seen in the fat body cells cultured for 24 hr, whereas the rate of their synthesis markedly increased, as seen in the cells from both sexes on the seventh day of cultivation (lanes 6 and 9). This is consistent with the developmental change of 30K protein mRNA shown in Fig. 4b.

It is known that the synthesis of plasma proteins in *B. mori* larvae is temporarily halted at the time of each molt, and

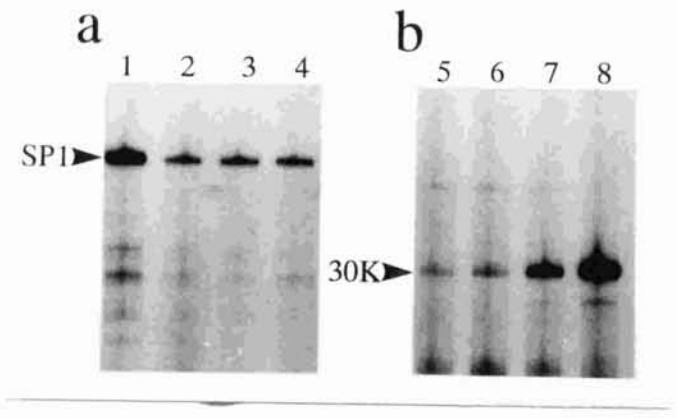
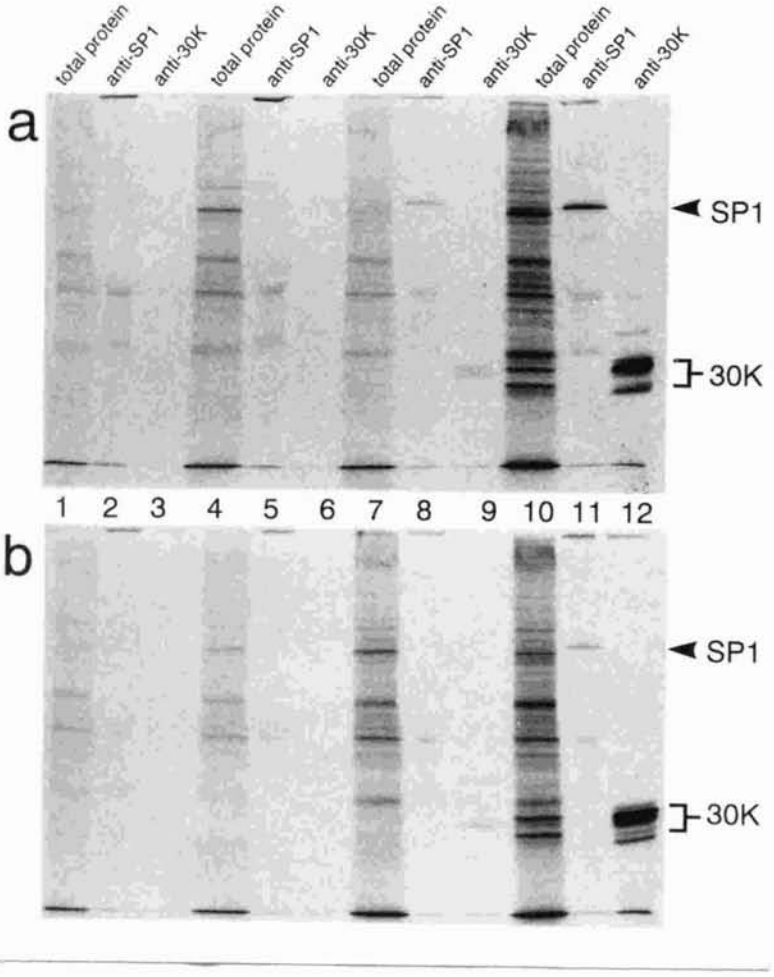


Fig. 4a, b. Developmental changes of SP1 and 30K protein mRNAs in primary cultures from female larvae on the fifth instar day-1. The fat body cells dissected from female larvae on the fifth instar day-1 were plated. Total RNA was prepared from the cultured cells immediately after seeding (lanes 1 and 5), 24 hr after seeding (lanes 2 and 6), on the third day of cultivation (lanes 3 and 7), and on the seventh day of cultivation (lanes 4 and 8). Each 3 μg RNA was annealed with the 5' end-labeled (1×10^5 cpm) SP1 primer 228 (a) or 30K6G1 primer 174 (b). Primer extension reactions were performed as described by Mine *et al.* (1995). Positions for extended fragments for SP1 and 30K protein mRNAs are indicated on the left of autoradiogramm.

feeding at the onset of intermolt stages triggers the resumption of protein synthesis in the fat body (Tomino 1985). To study the effect of feeding on the mode of plasma protein synthesis in the fat body cells of fifth instar larvae in culture, cells were isolated from the larval fat bodies just after the fourth molt, and protein synthesis was followed in the cultured cells. As is seen in Fig. 5, protein synthesis was negligible when the dispersed cells were put in culture (lane 1). In contrast to the fat body cells from the fifth instar day-1 larvae, a low level of protein synthesis persisted until the fourth day of cultivation (lanes 4 and 7). However, the rate of protein synthesis sharply increased in the cells of both sexes on the seventh day of cultivation, exhibiting sexually dimorphic expression of SP1 as well as marked stimulation of 30K protein synthesis as observed with normal fifth instar larvae (lanes 10, 11, and 12).


total protein
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b
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Fig. 5a, b. Plasma protein synthesis in primary cultures from female and male larvae just after the fourth molt. The fat body cells dissected from female (a) and male (b) larvae just after the fourth molt were plated. Cells were harvested on the day of seeding (lanes 1, 2, and 3), on the first day of cultivation (lanes 4, 5, and 6), on the fourth day of cultivation (lanes 7, 8, and 9), and on the seventh day of cultivation (lanes 10, 11, and 12). Proteins recognized with anti-SP1 (lanes 2, 5, 8, and 11) or anti-30K proteins (lanes 3, 6, 9, and 12) antibody were analyzed as described in "MATERIALS AND METHODS" and under Fig. 3. Lanes 1, 4, 7, and 10 represent total proteins synthesized in each preparation.

DISCUSSION

In the present study, I successfully established a primary culture for the fat body cells of *B. mori* that maintained their specific expression of plasma protein genes. The cultured fat body cells began to migrate to form cell cluster following adhesion to the surface of the culture dish. The structure of these clusters of fat body cells resembled that of fat body tissue. Oberlander (1976) observed that the dissociated fat body cells in suspension reaggregated; however, they did not form a fat body-like structure. As can be seen from Fig. 2, only part of the clustered cells synthesized SP1 and 30K proteins in this culture system. The observation may reflect the fact that fat bodies consist of heterogeneous cell types such as trophocytes, which carry out the broadest biochemical reactions (Haunerland and Shirk 1995), urate cells, which store uric acid (Buckner *et al.* 1985), and/or mycetocytes, which contain symbiotic bacteria (Houk and Griffith 1980). In fact, at least two types of cells were morphologically distinguishable in the primary culture of *B. mori* fat body cells. Additionally, previous studies suggest that fat bodies appear to be functionally separated into "biosyntheses type" and "storage type" in Lepidoptera (Haunerland *et al.* 1990; Wang and Haunerland 1992). It is not known whether cells expressing SP1 are identical to those expressing 30K proteins in this culture system. However, the percentage of immunoreactive cells in co-staining with both anti-SP1 and anti-30K protein antibodies was almost equal to that in staining with each antibody alone

(data not shown), suggesting that the same set of cells expresses both proteins.

The cultivated cells actively synthesized plasma proteins that include storage proteins and 30K proteins. In the primary culture developed from the fat bodies of fifth instar day-1 female larvae, the synthesis of SP1 was evident immediately after seeding. Both SP1 protein and SP1 mRNA synthesis were maintained at the same level throughout the culture period. The cells derived from the fifth instar day-1 males, on the other hand, hardly synthesized SP1 on the day of seeding. Although the rate of SP1 synthesis in male cells slightly increased during the period of the first to the seventh day of cultivation, the level was far lower than that observed for female cells. The observation indicates that irrespective of the medium milieu, the fat body cells per se are responsible for the sex-dependency of SP1 gene expression. This is in agreement with the previous finding that the sex-dependent expression of SP1 is genetically determined and developmentally regulated without participation of reproductive organs or any sex-specific humoral factors (Mine *et al.* 1983).

Easton and Horwath (1994) developed a primary cell culture system for *Tenebrio molitor* fat body in which the cells did not form cell clusters. In the present study, I show that synthesis of plasma proteins in the cultured fat body cells appears to be related with the change of cellular morphology. The synthesis of 30K proteins becomes apparent in the cells after the onset of cell clustering. The cells in suspension failed to synthesize 30K proteins. Furthermore, only the clustering cells continued to synthesize SP1 and 30K proteins (data not

shown). Several reports have shown that morphological changes of cultured cells are related with the activation of secretory protein genes. For example, it is known that mouse mammary epithelial cells accelerate the synthesis of milk proteins *in vitro* under cell clustering (Schmidhauser *et al.* 1990; Roskelley *et al.* 1994; Streuli *et al.* 1995). In view of these observations, it is likely that cell clustering is a prerequisite for plasma protein synthesis in cultured fat body cells. As shown in Fig. 3, the rate of total protein syntheses in the cells markedly increased on the first day of cultivation and then gradually declined. However, the rate of SP1 synthesis was constant, and that of 30K protein synthesis significantly increased during cultivation. Apparently, the regulatory mechanisms of plasma protein syntheses are different from those of other protein syntheses in this culture system.

Taken together, these results indicate that the fat body cells in primary cultures actively synthesize plasma proteins and faithfully reproduce the sex-dependency of SP1 synthesis. The long-term survival of functional cells in culture will certainly provide a useful system for gene transfection studies.

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Section II

Regulatory elements of major plasma protein genes in *Bombyx mori*

INTRODUCTION

In the silkworm, *Bombyx mori*, the fat bodies of actively feeding larvae synthesize plasma proteins and secrete them into hemolymph (Gamo 1978; Tojo *et al.* 1980; Tomino 1985). Major plasma proteins referred to as 30K proteins are a group of structurally related proteins with molecular weights around 30,000 (Sakai *et al.* 1988; Mori *et al.* 1991). These proteins are rather specific to the fifth (final) instar larvae. While negligible amounts of 30k proteins are detectable in hemolymph until the end of fourth larval instar, massive accumulation of these proteins in hemolymph takes place at the early final larval instar to pupal stage, and it has been shown that their synthesis in the fat body is developmentally regulated at the transcription level. These proteins are synthesized in the fat bodies of both female and male larvae and released into hemolymph, though large amounts of 30K proteins are detectable in oocytes of sexually matured females in the form of yolk proteins (Gamo 1978; Izumi *et al.* 1981). 30K proteins have a high homology in primary structure with microvitellogenin of *Manduca sexta*, which has been shown to be a female-specific yolk protein (Wang et al. 1988, 1989).

The onset of 30K protein synthesis is restricted to the early final instar larvae in concomitant with the disappearance of juvenile hormone (JH) from hemolymph (Gamo 1978; 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 ecdysteroid (Nijhout 1994). Both hormones induce a larval ecdysis and also plays an important role in almost every aspect of the development and reproduction of most insects by regulating the expression of specific genes (Dhadialla and Wyatt 1983; Bosquet and Calvez 1985; Nijhout 1994; Miura *et al.* 1998; Wyatt and Davey; Zhou *et al.* 1998a,b). In comparison with the knowledge for the mode of ecdysteroid action, little is known about the molecular mechanisms in which JH exerts its function. For the purpose of investigating the JH functions, I focused on the regulatory expression mechanisms of 30K protein genes.

In primary cultures of *B. mori* fat body cells, application of molecular biological approaches such as transfection has not previously developed. In this thesis, I established a method for introduction of DNAs into the fat body cells, and analyzed the 5' upstream region of 30K protein genes.

MATERIALS AND METHODS

Materials

A promoter/enhancer-less plasmid containing a luciferase reporter gene (PGV-B) and a luciferase assay reagent were purchased from Toyo ink. A cell lysis buffer, Passive Lysis Buffer, was purchased from Promega. Grace's insect medium (Grace 1962), TC100 insect medium (Ferkovich *et al.* 1994), and fetal bovine serum (FBS) were obtained from Gibco BRL. Dispase I (Matsumura *et al.* 1975a,b; Glyn and Brunt 1985) was obtained from Boehringer Mannheim. L-[^{35}S]-methionine was from American Radiolabeled Chemicals Inc. ENLIGHTNING was from Dupont/NEN.

All other chemicals were purchased from commercial sources.

Animals

The hybrid strain (*Kinshu x Showa*) of the silkworm, *Bombyx mori*, was obtained from Ueda Sanshu (Ueda, Nagano). Larvae were reared at 27°C on an artificial diet (Katakura Co.).

Hormone

Methoprene, a juvenile hormone (JH) analogue, was kindly supplied by Dr. S. Sakurai (University of Kanazawa). This analogue was stored at -20°C in glass vials. For use, dilution at 1:10000 (vol/vol) was made in ethanol using plastic pipet tips, and the dilution was added to TC100 insect medium containing 10% FBS at a final concentration of 100 ng/ml and mixed by vortexing (Dhadialla *et al.* 1987; Berger *et al.* 1992).

Protein synthesis in the cultured fat body cells

Aseptic dissections of *B. mori* larvae were performed as described in "Section I". In brief, the dorsal cuticle of the larva was cut longitudinally and pinned open on a silicone-rubber plate. Whole fat bodies from an individual larva were scraped away from the inside of the epidermal tissues and washed twice with Grace's insect medium. After the fat bodies were digested with 1.2 U/ml dispase I in the medium, the cells were washed twice with TC100 insect medium and once with the same medium containing 10% FBS. To study the effect of JH on the protein synthesis in the primary culture, the resultant cells were suspended in TC100 insect medium containing 10% FBS with methoprene or ethanol (a solvent for methoprene) as a control. The cells were plated onto a 96-well tissue culture plate (FALCON) and were maintained at 27°C in a humidified incubator. On the 5th day of cultivation, the cells were washed with phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM Na-phosphate buffer, pH 7.0) and incubated in 50 μl of PBS containing 30 μCi [^{35}S]-methionine for 3 hr. The radio-labeled cells were dissolved in 50 μl of 2 x SDS sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 250 mM Tris-HCl, pH 6.8) by heating for 5 min in boiling water. Electrophoresis was performed according to the methods of Laemmli using 10% acryl amide slab gel (Laemmli 1970). After electrophoresis, radioactive proteins were detected by fluorography using ENLIGHTNING.

Vector construction

The fusion gene p30KLUC was constructed as follows. The restriction fragment *Sac*I-*Sac*I (-1663 to +153) of *B. mori* 30K protein 6G1 gene (Mori *et al.* 1991) was inserted into the *Sac*I site of the plasmid PGV-B. The resultant plasmid was digested with *Mlu*I and *Xho*I to release a 706-bp fragment. This 706-bp fragment was replaced with a fragment, -553 to +14 of 30K protein 6G1 gene, which was amplified from the plasmid above by PCR using a 5' primer (5'-AAACAATCGAGTGACCTCAG) and a 3' primer (5'-TATCTCGAGTCACAACACTGTAGAAGC) including a new *Xho*I site at 3'-end and was digested with *Mlu*I and *Xho*I.

The plasmid p30KLUC was used to construct a series of 5' recurrent deletions of the promoter by combining restriction cutting and partial digestion by exonuclease as follows. The plasmid p30KLUC was cleaved at the *Afl*II site in the 30K protein gene insert and the *Kpn*I site in the vector DNA. The 5' deletion mutants, p30KLUCd848, d376, d176 and d11, were selected after exonuclease III digestion from the *Afl*II site of the large fragment to nucleotide (nt) position -848, -376, -176, and -11 bp, respectively.

The 5' deletion mutant p30KLUCd116 was constructed as follows. The fragments containing -116 to +14 of 30K protein 6G1 gene were amplified by PCR using a 5' primer (5'-ATACCCGGGCTTTGCAAATGACATCG) including a new *Sma*I site at 5'-end and a 3' primer (5'-GCCAAGCTTACTTAGATC). The PCR products were digested with *Sma*I and *Xho*I, and were inserted between the *Sma*I and the *Xho*I sites of the plasmid PGV-B.

The plasmid p30KLUCd48 resulted from insertion of the restriction fragment *Hinc*II-*Xho*I of the plasmid p30KLUC, which

contains -48 to +14 of 30K protein 6G1 gene, between the *Sma*I and the *Xho*I sites of the plasmid PGV-B.

The fusion gene pBmactA3LUC was constructed as follows. The fragments containing -127 to +160 of the A3 cytoplasmic actin gene of *B. mori* (Mangé *et al.* 1997) were amplified from the fat body DNA of *B. mori* (*Tokai x Asahi*) by PCR using a 5' primer (5'-AGCTGATAGCGTACGCGTTAC) including a new *Mlu*I site and a 3' primer (5'-TAAAGGGACGGAGAAGCTTCG) including a new *Hin*dIII site. The PCR products were digested with *Hin*dIII and *Mlu*I, and were inserted between the *Hin*dIII and the *Mlu*I sites of the plasmid PGV-B.

The fusion gene pLCP30LUC was constructed as follows. The restriction fragment *Hin*dIII-*Sac*I of the plasmid pLCP30CAT (Mine *et al.* 1995) was inserted between the *Hin*dIII and the *Sac*I site of the plasmid PGV-B. This plasmid contains the -1480 to +16 fragment of LCP30 gene.

Each plasmid DNA was purified by QIAGEN plasmid purification kit (QIAGEN).

Electroporation

Fat body cells used in this study were prepared from the larvae on the fifth instar day-1 of *B. mori* as described above. After dispase digestion, the fat body cells were washed three times with Grace's insect medium (serum-free). 4×10^6 cells suspended in 0.5 ml of the same medium were placed in a 0.4 cm gap electroporation cuvette (Bio-Rad) with the plasmid DNA. Electroporation was performed using a Gene Pulser Transfection Apparatus (Bio-Rad). Unless otherwise noted, transfection was administered at a single voltage of 90 V, a capacitance of 1075

μF and 1 pmol of DNA. The time constant of electroporation was typically 25 msec. After electroporation, the cells were washed once with the TC100 insect medium and suspended in TC100 insect medium supplemented with 10% FBS without antibiotics, and the cells divided into equal parts were plated onto a 60 mm-diameter tissue culture dish (FALCON) (2 x 10^6 cells in a dish). After incubation of the indicated periods at 27°C, the cells were assayed for luciferase activity. For the experiments of JH treatment, the cells in one dish were incubated in the presence of methoprene, and the cells in the other dish were treated with an equal volume of ethanol as a control.

All operations described above were performed under aseptic conditions.

Luciferase assay

The cells (2 x 10^6 cells) were scraped from the dish with a rubber policeman and washed twice PBS and resuspended in 50 μl of lysis buffer for 15 min at room temperature. The lysate was centrifuged at 12,000 x *g* for 2 min at 4°C. 100 μl of luciferase assay reagent was transferred to a luminometer cuvette, and the luciferase activity in each sample was assayed immediately after 20 μl of the cell lysate was added to the reagent in the cuvette.

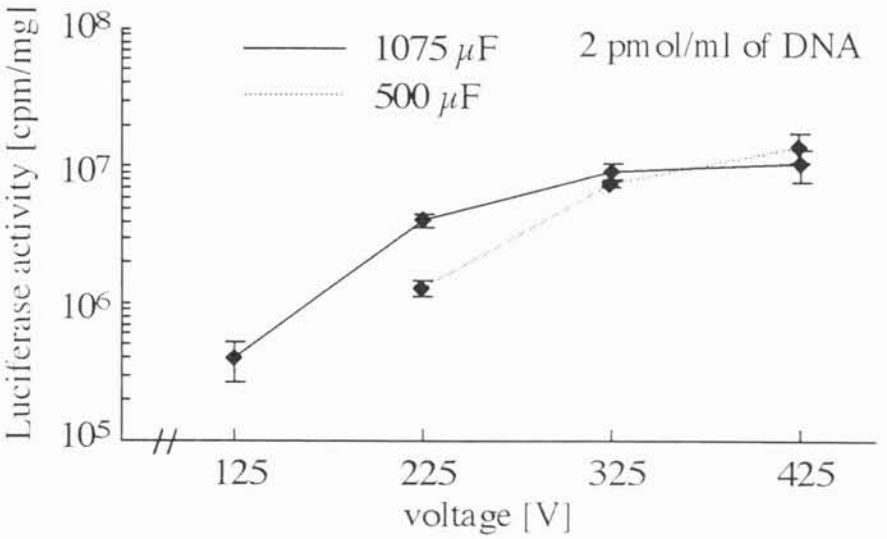
Luciferase assay was normalized by the concentration of protein in each lysate, which was determined according to the method of Bradford (1976). Each determination of luciferase activity presented in "Results" is an average of three assays with its associated standard error.

RESULTS

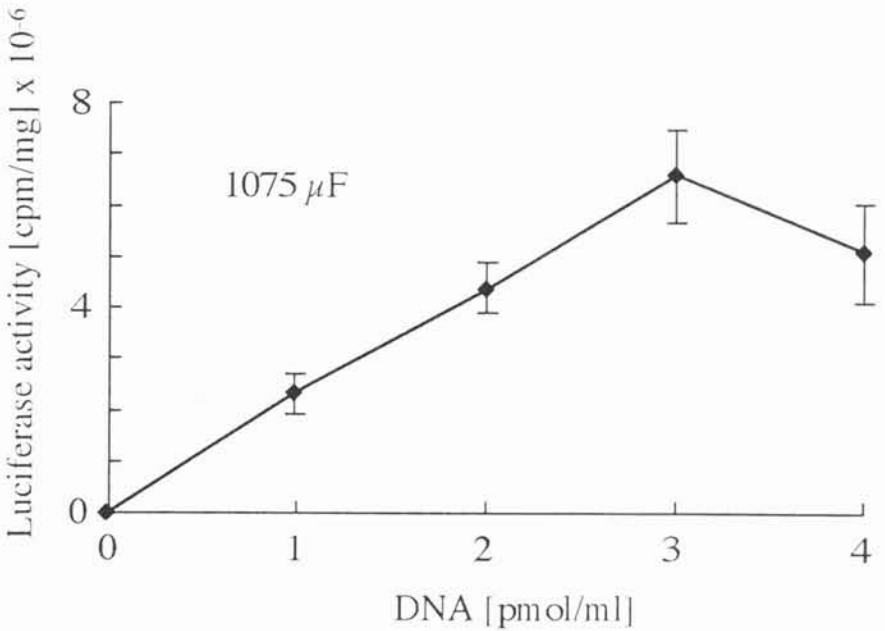
Optimization of transfection condition in B. mori fat body cells

To establish an assay system using cultured fat body cells for the transcriptional regulation of plasma protein genes, I attempted various transfection methods including calcium phosphate (Graham and van der Eb 1973), DEAE dextran (McCutchan and Pagano 1968), and liposome (Fraley 1980) methods. But, I could not detect any activities derived from reporter genes in the systems above. Finally, I adopted electroporation method (Chu *et al.* 1987) to introduce DNA into the cultured cells. Consequently, I could successfully detect the luciferase activity in the fat body cells after introducing the plasmid p30KLUC, which has the promoter sequence of 30K protein 6G1 gene joined with the luciferase gene as reporter gene. The luciferase activity was sufficiently detected in the cell lysate from the application of 4×10^6 fat body cells using Grace's insect medium as electroporation buffer. I investigated optimal conditions of electroporation for introduction of the plasmid p30KLUC into cultured fat body cells. Altering voltage, capacitance, DNA concentration, and cultured periods after transfection resulted in a range of luciferase activities which varied two orders of magnitude (Fig. 1). Effects of voltage and capacitance on the transfection into fat body cells are shown in Fig. 1a. Luciferase activity in transfected cells increases until 325 V/cm and reached plateau around 425 V/cm at 1075 μF. On the other hand, at 225 V/cm, luciferase activity at 500 μF was lower than that at 1075 μF, though the activities above 325 V/cm are nearly same level as

a.


Luciferase activity [cpm/mg]
10^8
10^7
10^6
10^5
1075 μF
500 μF
2 pmol/ml of DNA
125
225
325
425
voltage [V]

b.


Luciferase activity [cpm/mg] x 10^{-6}
8
4
0
1075 μF
0
1
2
3
4
DNA [pmol/ml]

c.

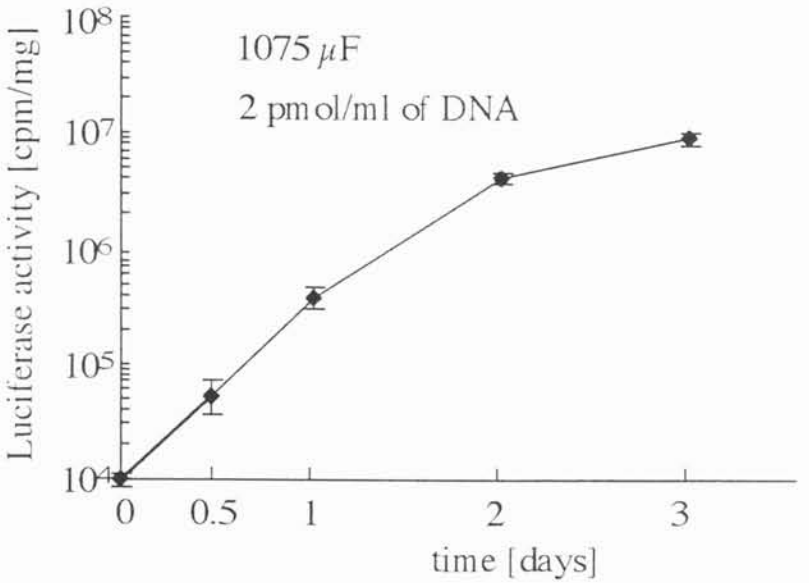

Luciferase activity [cpm/mg]
10^8
10^7
10^6
10^5
10^4
1075 μF
2 pmol/ml of DNA
0
0.5
1
2
3
time [days]

Fig. 1a-c. Analyses of various electroporation conditions. Each luciferase activity was normalized per 1 mg of total proteins in each lysate. Bars represent standard errors of n=3. a; Electroporation as functions of voltage and capacitance. The fat body cells (4×10^6 cells) suspended in 0.5 ml of Grace's insect medium were electroporated with 1 pmol of the plasmid p30KLUC at a range of 50 V to 170 V in a 0.4 cm cuvette and two different capacitances, 1075 μF (a solid line) and 500 μF (a dotted line). 2×10^6 cells were plated per 60 mm dish. Cell extracts were harvested on the 2nd day of cultivation and assayed for luciferase activity. Luminescence is plotted on the y-axis using a log scale. b; The effects of DNA concentration on luciferase activity. The fat body cells were electroporated with different concentrations of the plasmid p30KLUC at 90 V and 1075 μF in a 0.4 cm cuvette. The cells were harvested on the 2nd day and cell extracts were assayed for luciferase activity. c; Time course of luciferase activity after electroporation. The fat body cells were electroporated with 2 pmol/ml of the plasmid p30KLUC at 90 V and 1075 μF in a 0.4 cm cuvette. Cell extracts were assayed at various times for luciferase activity. Luminescence is plotted on the y-axis using a log scale.

those at 1075 μF. The cells exposed to the electric field below 225 V/cm adhered on the surface of culture dish and clustered after plating, while the cells exposed voltage above 325 V/cm adhered little on the dish. So, I adopted 225 V/cm and 1075 μF as optimum conditions of voltage and capacitance, respectively.

Fig. 1b shows the correlation between DNA concentration in the electroporation buffer and luciferase activity in the transfected cells. The activity increased in proportion to the DNA concentration up to 3 pmol/ml, then decreased at 4 pmol/ml. The time course of luciferase activity after electroporation is also shown in Fig. 1c. Luciferase activity sharply increased until 2 days after electroporation and reached maximal on the day-3 of cultivation. So, I settled the time to harvest the cells on the 2nd day after electroporation, since the transfected cells on the 3rd day of cultivation adhered too tight to be scraped completely, though the higher luciferase activity occurred on the day-3.

For reason mentioned above, I, hereafter, performed electroporation at 225 V/cm, at 1075 μF and 1 pmol of DNA in 0.5 ml of serum-free Grace's insect medium as an electroporation buffer containing 4 x 10^6 fat body cells and the transfected cells were harvested on the 2nd day of cultivation.

Promoter specific effects in B. mori fat body cells

Using the optimized conditions mentioned above, I analyzed the promoter activities of three different *Bombyx* genes, 30K protein 6G1, cytoplasmic actin A3 and LCP30 genes, to drive a luciferase reporter gene in fat body cells (Fig. 2). The

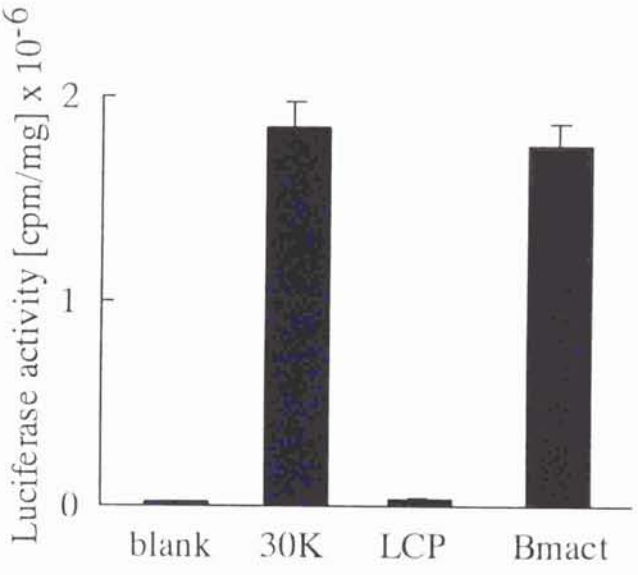


Fig. 2. Luciferase activity in *B. mori* fat body cells on transfection with each plasmid containing different promoters. The fat body cells were electroporated with 2 pmol/ml of the plasmid p30KLUC (30K), pLCP30LUC (LCP), or pBmactA3LUC (Bmact) under the optimized condition. Cell extracts were harvested on the 2nd day of cultivation and assayed for luciferase activity. These results are shown at mean of three experiments with its associated standard error.

plasmid p30KLUC contains the 5' upstream sequence of 30K protein 6G1 gene, which is fat body-specific gene, and the plasmid pBmactA3LUC contains the promoter region of ubiquitous cytoplasmic actin A3 gene. These plasmids generated the high luciferase activity in the transfected cells. On the other hand, the plasmid pLCP30LUC, containing the 5' upstream region of the LCP30 gene, a larval epidermis-specific gene (Nakato *et al.* 1994), generated negligible luciferase activity. These results clearly show that the promoter-reporter gene constructs introduced into fat body cells exhibit highly regulated tissue-specific expression.

Analysis of the regulatory elements in 30K protein gene promoter

To clarify the role of the 5' upstream region of the 30K protein 6G1 gene in transcription, a series of the 5' deletion mutants were constructed from p30KLUC as described under "MATERIALS AND METHODS". The master plasmid p30KLUC and the deletion constructs were introduced into the cultured fat body cells and the luciferase activities were assayed after cultivation. The test of significance among each of the data was performed by Tukey method (1% as a standard) using SAS system (PROC ANOVA). There was no significance between the data from deleted mutants of −848 and −176, or −48 and −11 in Fig. 3, respectively. Significance existed among each of the data from other constructs.

When the construct deleted to nt position -848 was introduced into the fat body cells, the luciferase activity increased about twice compared with the result of introducing p30KLUC (-1668 construct in Fig. 3b). It is likely that

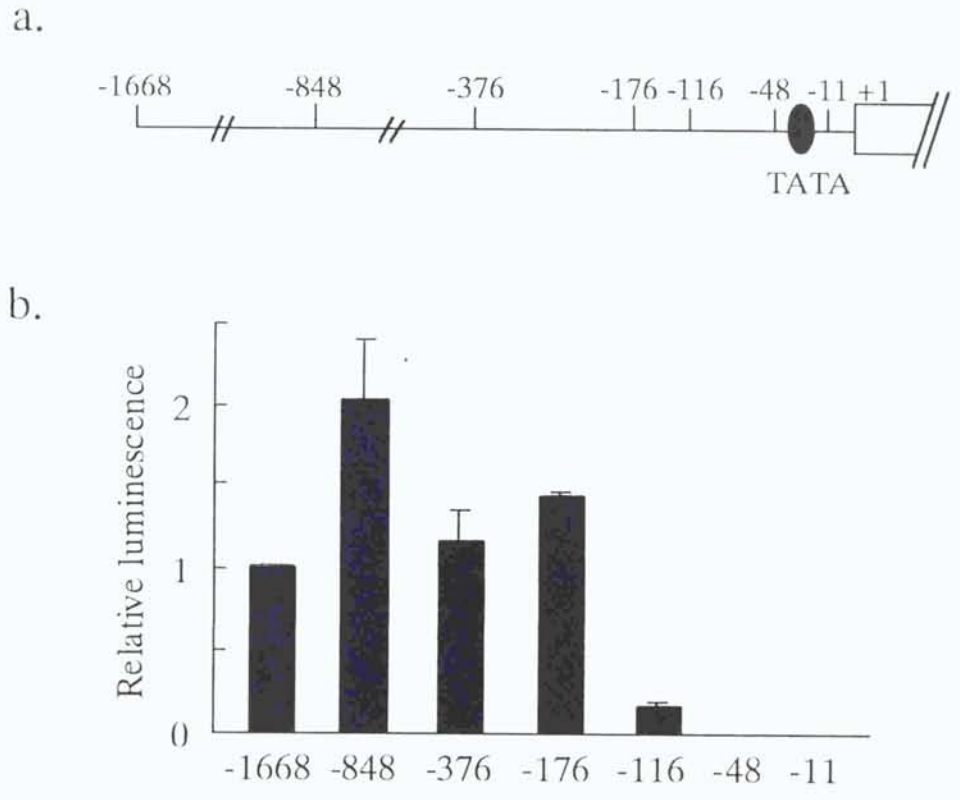


Fig. 3. Expression analysis of p30KLUC constructions. 5' Deletion mutants of p30KLUC are shown in Fig. 3a with end-point positions of each deletion. The fat body cells were electroporated with 2 pmol/ml of each deletion mutant and the luciferase activities were assayed on the 2nd day after electroporation. The results are given relative to full promoter activity (p30KLUC).

silencer-like element exists in the region between nt position -1668 and -848. The activity of luciferase kept at high levels when the mutants construct lacking various length of the 5' upstream region between -848 and -176 were introduced in the fat body cells. However, deletion extending to nt position -116 led to marked decrease in the luciferase activity. These results strongly suggest that some enhancer elements for tissue-specific expression of 30K protein 6G1 gene exist between nt position -176 and -116. The further deletion extending to nt position -48, which is 18 bases upstream of TATA box, led to the loss of luciferase activity, showing that the sequence between nt position -116 and -48 includes some basal regulatory elements for the transcription. These results indicate that some contiguous nucleotide sequences existing upstream of 30K protein 6G1 gene play positive roles in expression of the fat body-specific gene.

Analysis of the JH-responsive element

Previous study indicated that the synthesis of 30K protein is negatively regulated by JH *in vivo* (Izumi *et al.* 1984). To confirm that the fat body cells in the primary culture also respond to JH, the cells were incubated in the presence of a JH analogue, methoprene, and the protein syntheses in the methoprene-treated cells were analyzed on the 5th day of cultivation. As shown in Fig. 4, the cells treated with methoprene hardly synthesized any 30K proteins, while the syntheses of other proteins were not affected by methoprene. So, I attempted to treat the fat body cells with methoprene after introduction of the promoter construct of 30K protein 6G1 gene.

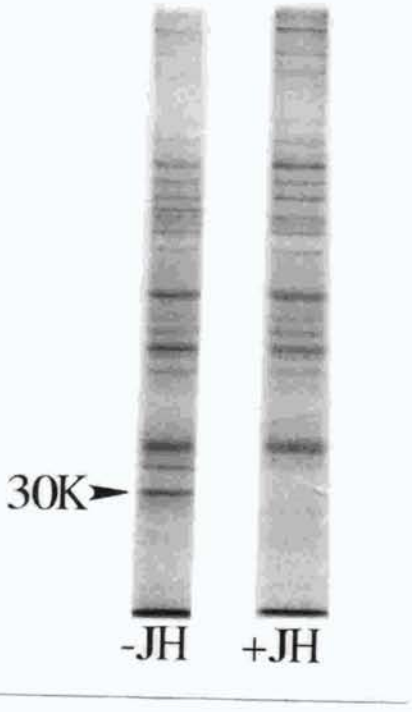


Fig. 4. Effects of JH on protein syntheses in the primary culture of the fat body cells. The fat body cells prepared from female larvae on day-1 of the fifth instar were cultured in the presence (+JH) or absence (-JH) of methoprene as described in "MATERIALS AND METHODS". Each cell preparation was incubated with 50 μl of PBS containing 30 μCi [^{35}S]-methionine for 3 hr at 27°C. The cells were dissolved in each 50 μl of SDS sample buffer and total proteins in each preparation were electrophoresed and the labeled proteins were detected by fluorography.

The test of significance among each of the data was also performed using SAS system as above.

As depicted in Fig. 5, methoprene-treated fat body cells after introduction of the plasmid p30KLUC remarkably decreased the luciferase activity approximately to 30% as compared with control experiment, in which the transfected cells were treated with ethanol, solvent of methoprene (-1668 in Fig. 5). By contrast, the luciferase activity in the cells transfected with the pBmactA3LUC was not affected by the methoprene-treatment (Bmact in Fig. 5). Subsequently, to analyze the JH-responsive element lying on the 5' upstream sequence of 30K protein 6G1 gene, I treated the fat body cells after introduction of a series of deletion mutants with methoprene and assayed luciferase activity in the cell lysates. Methoprene-treatment of the fat body cells transfected 5' deletion constructs from nt position -1668 to -176 led to decrease luciferase activities to approximately 30% as compared with each control. There was no significance among each of the data from deleted mutants of -1668 to -176 in the presence of methoprene. Deletion extending to nt position -116, however, led to reduce the sensitivity for methoprene. The luciferase activity in the methoprene-treated cells transfected the deletion construct to nt position -116 was 60% of that of control experiment (-116 in Fig. 5). The result of the test of significance supports the existence of significance between the data from -116 construct and other constructs in the presence of methoprene. These results show that some contiguous nucleotide sequences being upstream of 30K protein 6G1 gene respond to JH and play negative roles in expression of the fat body-specific gene in the presence of JH.

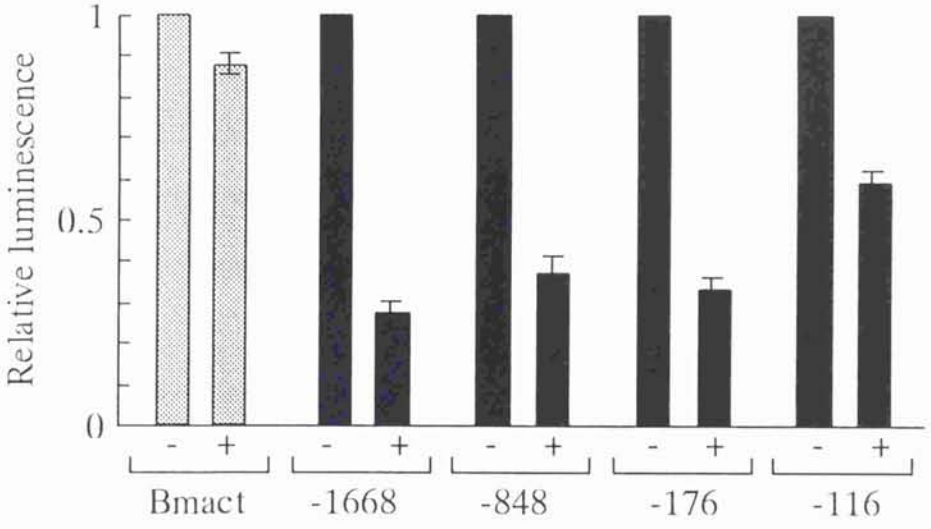


Fig. 5. Effects of JH on luciferase activity in each cell transfected with 5' deleted promoter constructs of 30K protein 6G1 gene. The fat body cells were electroporated with 2 pmol/ml of each deletion mutant then cultured in the presence or absence of methoprene. The cells were harvested on the 2nd day of cultivation and cell extracts were assayed for luciferase activity. The results are given as the luciferase activity from the cells in the presence of methoprene relative to that from the cells in the absence of one.

DISCUSSION

Major plasma proteins termed 30K proteins are synthesized by the fat body cells of the silkworm, *Bombyx mori*, in a tissue- and stage-specific manner during the larval development (Gamo 1978; Izumi *et al.* 1981; Sakai *et al.* 1988; Mori *et al.* 1991). To investigate the molecular mechanisms underlying expression of plasma protein genes, the cell-free transcription systems have been developed from the nuclear extract of the larval fat body cells, which preferentially transcribed a homologous storage protein gene in a promoter specific manner (Mine *et al.* 1995). However, it is difficult to completely reproduce the specificities of plasma protein gene expression in the cell-free system. In this section, an alternative attempt for the analysis of expression of plasma protein genes was made to introduce the gene constructs into the primary cultured cells of the *B. mori* fat body. Transfection approaches of DNAs into primary cultured cells have seldom been adopted for almost every animal, containing the silkworm, *B. mori*. However, I finally succeeded to introduce DNAs into the primary cultured fat body cells of *B. mori* using an electroporation technique (Chu *et al.* 1987). Quon *et al.* (1993) have already developed a transfection method of DNAs into isolated rat adipose cells by electroporation. They reported that six shocks at 800 V and 25 μF in 0.4 cm cuvette resulted in efficient transfection. On the other hand, optimum conditions to introduce DNA into the cultured fat body cells of *B. mori* are one shock at 90 V and 1075 μF in 0.4 cm cuvette.

The fat body cells of *B. mori* in primary culture actively synthesize plasma proteins such as storage proteins and 30K proteins (Section I in this thesis). As shown Fig. 2, each gene construct introduced into fat body cells was also expressed in tissue-specificity manner. Namely, the 30K protein and the actin promoter constructs revealed high luciferase activity in the cultured fat body cells, the other hand, the promoter of LCP30 gene, which expresses in the larval epidermis, had little transcription activity in the cells. This result shows that introduction of DNA into the primary cultured cells of the fat body is an efficient system to study the regulatory mechanism of expression of fat body-specific genes, including plasma protein genes. Subsequently, I analyzed the effect of the 5' upstream sequence of 30K protein 6G1 on the transcription. The analysis showed that some enhancer elements located between nt position −176 and −48 from the transcription initiation site of the 30K protein 6G1 gene. Previously, Mori (1992) showed that at least three nuclear proteins in the fat body cells of early fifth instar larvae bound to the sequence between nt position -186 to -49 of 30K protein 6G1 gene by a gel shift assay. The computational analysis of the cis-acting elements using TF search program revealed the consensus binding sequences of many transcriptional factors, including broad complex (BRC) and CCAAT displacement protein (CDP). BRC is one of early gene products induced by ecdysteroid (Andres *et al.* 1993; Thummel 1996). BRC might be one of candidates for transcription factors of 30K protein genes, because ecdysteroid elevates the rate of 30K protein synthesis in the organ cultured fat body from the fifth instar larvae (Izumi 1981). CDP is one of the CCAAT-

binding factors in vertebrates (Superti-Furga *et al.* 1989; Skalnik *et al.* 1991). It is possibly that a *Bombyx* homologue of CDP raises the basal transcription level of 30K protein gene.

The physiological functions of JH are well studied using a powerful JH analogue, methoprene, but the molecular mechanisms of JH action are little known. One of the most crucial reasons is that the JH receptors have not been identified yet. On the other hand, the JH binding proteins, which circulate the JH in insect hemolymph, have been identified and characterized (Trowell 1992). It is also known that JH participates in the regulation of expression of many genes (Dhadialla and Wyatt 1983; Bosquet and Calvez 1985; Miura *et al.* 1998), but the JH-responsive elements of each gene have not been identified yet. The delay of research on mechanisms of JH action as above is due to the lack of several key experimental capabilities.

Izumi *et al.* (1984) reported that the synthesis of 30K proteins in *B. mori* seemed to be suppressed by JH. In this study, I clearly showed that methoprene completely suppressed the expression of 30K protein gene in the primary culture system of fat body cells. Subsequently, I performed a series of experiments to clear the location of JH-responsive elements in the 5' upstream region of 30K protein 6G1 gene. As shown in Fig. 5, the JH-responsive element of 30K protein 6G1 gene localized downstream of nt position −176, just overlapping with its enhancer sequence. These results indicate that the expression of 30K protein 6G1 gene is regulated by the competitive binding of the transcriptional activators and the JH-responsive transcriptional repressors to the downstream

sequence of nt position -176. BRC might be a candidate of transcriptional factors controlling the expression of 30K protein 6G1 gene as mentioned above. It is known that expression of BRC gene is regulated by not only ecdysteroid but also JH (Restifo and Wilson 1998; Zhou *et al.* 1998b).

In this study, I provided a new and powerful approach to analyze the cellular and molecular modes of JH action. This method is fairly helpful to make clear the JH-mediated gene regulation, including to identify the nuclear receptors of JH.

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