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The fat body cell-free system for tissue-specific transcription of plasma protein gene of *Bombyx mori*

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ABSTRACT

A nuclear extract was prepared from the larval fat body of the silkworm, *Bombyx mori*, and a homologous *in vitro* system was developed for the transcription of major plasma protein gene of *B.mori*. The gene for SP1, a storage protein of *B.mori*, and adenovirus 2 major late (AdML) gene were faithfully transcribed under relatively high template concentrations in the nuclear extract prepared from the fat body of female fifth instar larvae. Complete inhibition of gene transcription by a low concentration of α-amanitin indicated that the reaction is catalyzed by RNA polymerase II. At low template concentration (0.6 nM) the fat body nuclear extract transcribed the homologous SP1 gene with high efficiency, while AdML gene and larval cuticle protein gene were only barely transcribed in the same extract. The SP1 gene deleted upstream of the TATA box sequence showed little effect on transcription, whereas mutations that destroy TATA sequence totally abolished the gene transcription. These results suggested that the core promoter region of SP1 gene spanning between positions –44 and +16 is essential for the fat body specific transcription *in vitro*.

INTRODUCTION

Recent advancement in technology of analyzing gene transcription in cell-free systems has greatly contributed new knowledge for our understanding of the molecular processes involved in the regulation of eukaryotic gene transcription (1–9). Transcriptional activation of eukaryotic genes involves the regulated assembly of multiprotein complexes on enhancer and promoter sequences (10,11).

We have been interested in the mechanisms of specific gene activation during insect metamorphosis and working on the structures and expression of major plasma protein genes of the silkworm, *Bombyx mori*. The major plasma proteins of *B.mori* that include a storage protein termed as 'SP1' are synthesized by the fat body cells and secreted into hemolymph in a stage- and sex-dependent fashion during post-embryonic development (12–14). Our previous studies have provided evidence that the biosyntheses of these plasma proteins are regulated at the level of gene transcription in the fat body cells (13,14). Thus, by exploring the processes of regulated transcription of major plasma protein genes, it would be feasible to gain an insight into molecular events underlying insect metamorphosis. As an initial step toward the purpose, we attempted to develop a homologous cell-free transcription system from the fat body cells. In this paper we describe the method for successful preparation of a tissue-specific *in vitro* transcription system from the larval fat body of *B.mori* and show that the core promoter region of SP1 gene plays a role in specific transcription *in vitro*.

MATERIALS AND METHODS

Animals

Larvae of a commercial strain of the silkworm, *B. mori* (*Kinshyu* × *Showa*) were obtained from Kyodo Shiryo Co. and reared on an artificial diet (Yakult Co.) at 25°C.

Template DNAs, S1 probes and primer DNAs

The following plasmids were used as the templates for transcription reactions. Construction and structures of plasmid containing AdML gene (pAd), the hybrid plasmid consisting of 5′ upstream region of the SP1 gene and the chloramphenicol acetyl transferase (CAT) gene (pSP1CAT1800) and its deleted mutants have been described previously (16). pSP1XScat was constructed as follows: an *Xho*I–*Sal*I fragment [nucleotide (nt) positions –1150 to +2400 of the SP1 gene was subcloned into pUC9. A *Sac*II–*Xcm*I sequence (nt positions +58 to +108) in the first exon of SP1 gene was deleted and replaced with the synthetic oligonucleotide shown below bearing a portion of the CAT structural gene.

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5′-GGCGTCAGCCTCAGCGATTAGTGGTGGCAAGCTTAACCAGACCGTTCAGCTGGAA-3′
3′-CGCCGCAGTCGGAGTCGCTAATCACCACCGTTCGAATTGGTCTGGCAAGTCGACCT-5′
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LCP30CAT is a hybrid plasmid containing the –1500 to +16 fragment of a larval cuticle protein (LCP30) gene of *B.mori* fused with the CAT gene (15).

The ^{32}P-labelled probe DNAs used for S1 nuclease protection analyses of the transcripts were prepared as described previously (16).

Nucleotide sequences of primers for reverse transcription analysis of the *in vitro* transcripts encoded by CAT chimeric genes

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and AdML template were 5′-TCCAGCTGAACGGTCTGG-TT-3′ and 5′-CTGACCAGATGGACGCGGCC-3′, respectively. These oligonucleotides were synthesized by use of a Milligen/Biosearch DNA Synthesizer, and labelled with ^{32}P at 5′ ends as described (13).

Preparation of crude nuclear extract from fat body

The fifth instar day-2 larvae were decapitated by tearing the integument and viscera were squeezed out with fingers. Fat bodies inside of the integument were wrung out with a glass spatula, frozen immediately under liquid nitrogen and stored at –80°C until use.

All the following procedures were carried out at 0–4°C. Frozen fat bodies were thawed in 5 vol of 10 mM HEPES–KOH (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 0.2% NP-40 (buffer A), and the suspension was placed on ice for 10 min. Swollen fat bodies were pelleted by centrifugation at 1000 *g* for 5 min. The fat bodies were resuspended in 3 packed-tissue volumes of buffer A and homogenized by 10 strokes with a Dounce homogenizer using a tight-fitting pestle. The homogenate was filtered successively through two layers of stainless wire screens with mesh apertures 212 and 106 μm, respectively (Tokyo Screen Co.). Debris retained by the screens was rinsed with 2 packed-tissue volumes of buffer A. The combined filtrate was centrifuged at 1500 *g* for 5 min. The supernatant was removed with aspirator and lipid deposits on the side of the centrifuge tube were cleaned with Kimwipes. The tube was centrifuged again at 25 000 *g* for 20 min to pack the nuclei. The supernatant was discarded completely and the pelleted nuclei were subjected to salt-extraction as described previously for the preparation of nuclear extract from the BmN cells (16). Protein concentration of each extract was determined according to the method of Bradford (17).

Preparation of extract from BmN cells and posterior silk glands

BmN whole cell extract was prepared as described previously (16).

The nuclear extract from posterior silk glands (PSG) was prepared according to the method of Suzuki and Suzuki (18) except that the ammonium sulfate precipitate was dissolved in DB [20 mM HEPES–KOH (pH 7.9), 0.2 mM EDTA, 0.4 mM PMSF, 1 mM DTT, 15% (v/v) glycerol] containing 0.1 M KCl and dialyzed against the same buffer.

In vitro transcription

In vitro transcription reaction was carried out for 1 h at 20°C in a 25 μl mixture containing 16 mM HEPES–KOH (pH 7.9), 60 mM KCl, 4 mM $MgCl_2$, 0.16 mM EDTA, 0.8 mM DTT, 11% (v/v) glycerol, 0.5 mM each of ATP, CTP, UTP, GTP and 2500 U/ml RNase inhibitor which we purified from human placenta according to the method of Blackburn (19). Template DNA and the nuclear extract were added at concentrations given in the figure legends. The reaction was terminated by the addition of an equal volume of 10 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 50 μg of the bulk mixture of wheat germ tRNA (Sigma Chemical Co.), 5 μg DNase I (Worthington Biochemical Co.). After incubation for 30 min at 37°C, 150 μl of a solution containing 20 mM EDTA, 0.1 M sodium acetate (pH 5.5), 1% SDS was added to the mixture. The mixture was extracted twice with phenol/chloroform (1:1, v/v), and the RNA transcripts were recovered by ethanol precipitation.

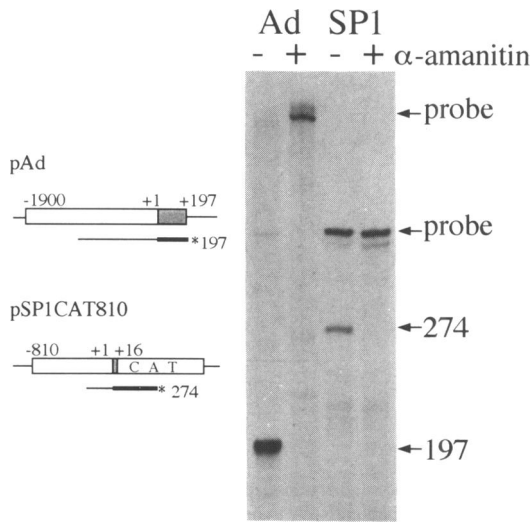

Figure 1. *In vitro* transcription of adenovirus and *B.mori* genes in the fat body nuclear extract. Diagrams of templates and S1 probes are shown to the left of the radioautogram. pAd and pSP1CAT810 indicate template plasmids containing the gene for adenovirus major late protein and a hybrid plasmid consisting of 5′ upstream region of SP1 gene and CAT structural gene, respectively. Open boxes represent the DNA inserts, and thin lines show vector DNA regions. +1 indicates transcription initiation sites and shaded areas are exons. Solid lines with asterisks under each template show the end-labeled probe DNAs for S1 nuclease protection analyses, and bold lines show regions supposed to be protected against S1 nuclease digestion. Transcription was performed in reactions each containing 4 nM of pAd or pSP1CAT810 template and the fat body nuclear extract (50 μg protein) prepared from the female fifth instar larvae. RNA transcripts were analyzed by S1 nuclease protection analysis as described in 'Materials and Methods'. Reactions were carried out with (+) or without (–) 1 μg/ml α-amanitin. Arrows with numbers indicate the sizes in bases of the protected fragments.

Analysis of RNA transcripts *in vitro*

RNA synthesized *in vitro* was quantitated by S1 nuclease protection or primer extension analysis.

For S1 nuclease protection analysis, RNA transcripts were dissolved in 30 μl of 80% formamide, 0.4 M NaCl, 40 mM PIPES–NaOH (pH 6.4), 1 mM EDTA and the 5′ end labelled DNA fragments (3×10^4 c.p.m.). The solution was incubated at 85°C for 5 min, and then at 37°C for 4 h. After treatment with 100 U/ml of S1 nuclease (Takara Shuzo Co.) as described (13), the protected fragments were recovered by isopropanol precipitation.

For primer extension analysis, RNA transcripts were annealed with the 5′ end labeled primer DNA (2×10^5 c.p.m.) in a 10 μl solution containing 20 mM Tris–HCl (pH 8.0), 0.2 mM EDTA, 0.2 M KCl at 65°C for 5 min, then at 55°C for 20 min, and cooled to room temperature. The annealed primer was extended with 5 U of Avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences Inc.) at 42°C for 1 h in 20 μl of 0.1 M Tris–HCl (pH 8.3) 10 mM $MgCl_2$, 10 mM DTT, 0.1 M KCl, 50 μg/ml actinomycin D and 2500 U/ml human placental RNase inhibitor. The reaction was stopped by adding 50 μl ethanol and nucleic acids were precipitated by centrifugation.

The nucleic acid pellets were separated on 6% acrylamide–7 M urea gel and radioactivity was detected by autoradiography. In some experiments, the bands corresponding to the specific transcripts were

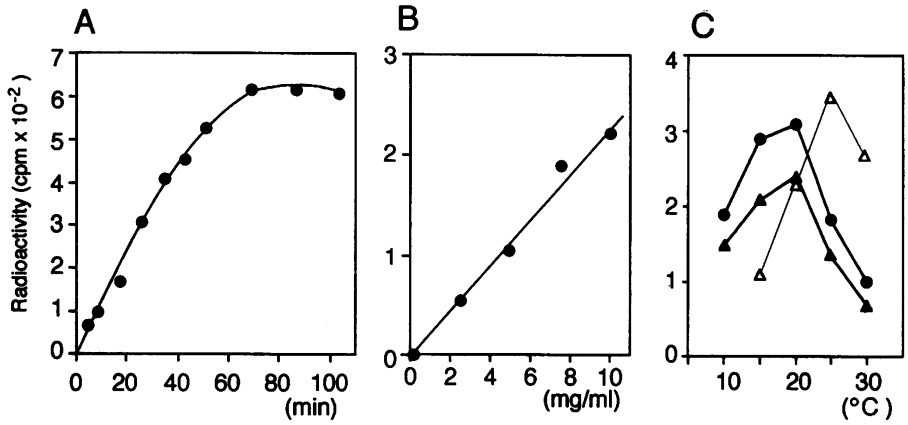

Figure 2. Conditions for *in vitro* transcription. Transcription was performed under standard condition in the presence of each 4 nM of template and nuclear extract as indicated. After incubation, RNA transcripts were analyzed by primer extension followed by gel electrophoresis and autoradiography. The amounts of specific transcripts were quantitated as described in 'Materials and Methods'. (**A**) Time course of transcription reaction. Reaction was performed on pSP1CAT810 template in the presence of each 50 μg protein/ml of fat body nuclear extract from the female fifth instar larvae. (**B**) Effect of nuclear protein concentration. Transcription reaction was run on pSP1CAT810 for 1 h in the presence of the indicated concentration of fat body nuclear extract from the female fifth instar larvae. (**C**) Temperature dependency. Transcription reaction at indicated temperature was performed in the presence of 50 μg protein/ml of fat body nuclear extract from the female fifth instar larvae on pSP1CAT810 (closed circles) or pAd (closed triangles). Open triangles indicate temperature dependency of transcription reaction determined on pAd template in the presence of BmN whole cell extract (75 μg protein/ml).

excised from the dried gel and radioactivity was determined by Cerenkov-counting.

RESULTS

Preparation of nuclear extract from fat body

Since the fat body cells of *B. mori* are highly rich in lipid materials and the tissues are surrounded by tight basement membrane, special treatments are required to obtain nuclei with high yield. After several trials, we found that swelling the fat body tissue in a hypotonic buffer prior to homogenization greatly improves the yield of nuclei. Hence, the fat bodies were swollen in a low-salt buffer as detailed in 'Materials and Methods' and homogenized with a Dounce homogenizer, which completely disintegrated the basement membrane and yielded intact nuclei. Addition of NP-40 at final concentration of 0.4% to the homogenizing buffer helped to separate nuclei readily by centrifugation from lipid substances stored in fat body cells. The fat body nuclei thus isolated were extracted with a high salt buffer following the procedure described by Sakurai *et al.* (16). We normally obtained 6 mg protein from 1.5 ml packed volume of crude nuclei isolated from the fat bodies of 100 larvae.

In vitro transcription by RNA polymerase II

Transcriptional activity of the nuclear extract was assayed on the adenovirus 2 major late (AdML) gene template which is known to be transcribed efficiently *in vitro* even in heterologous systems. The SP1 gene which encodes the *B.mori* storage protein was used as a homologous template DNA. Our previous study has indicated that transcription of the SP1 gene is most prominent in the fat body of females at early fifth larval instar (13). Figure 1 shows the result of S1 nuclease protection analyses of the *in vitro* transcripts. When the template plasmid containing AdML gene (pAd) was transcribed at a template concentration of 4 nM in the fat body nuclear extract and the products were analyzed by S1 nuclease mapping, a 197 base DNA band was detected as a fragment protected against S1 nuclease digestion. Similarly, a 274 base fragment was observed by the S1 nuclease protection analysis of the transcripts encoded *in vitro* by the same concentration of pSP1CAT810 plasmid DNA containing the SP1 gene promoter. The sizes of these protected fragments comply with the lengths from the radiolabelled ends to the transcription initiation sites of pAd and pSP1CAT810, respectively. The appearance of these protected bands was completely abolished by the presence of 1 μg/ml α-amanitin in each reaction mixture. The result demonstrates that the *B.mori* fat body nuclear extract can accurately transcribe both the AdML and SP1 genes, and that the transcription reaction is dependent on RNA polymerase II.

Optimal conditions for *in vitro* transcription

Optimal conditions for *in vitro* transcription in the fat body system was determined on pSP1CAT810 and pAd template. The amount of SP1 transcript synthesized increased linearly with time up to 80 min (Fig. 2A). The rate of transcription on SP1 template incereased in proportion to the nuclear protein concentration up to 10 mg/ml (Fig. 2B). Figure 2C shows that transcription was maximal when the reaction was carried out at 20°C. *In vitro* transcription reactions using extracts from the cultured insect cells (2,7), *Drosophila* embryo (5,20) and posterior silk glands of *B.mori* (1) exhibit broad temperature optima ranging from 21 to 30°C. Thus, each system appears to have different property with respect to temperature dependency. The optimal concentrations of K^+ and Mg^{2+} for transcription were found to be 60 and 4 mM, respectively.

Tissue specific transcription of SP1 gene

Previous studies have indicated that the rate of SP1 gene transcription in the fat body cells is nearly equal in both sexes until the end of fourth larval instar, whereas in the fifth instar larvae, the amount of SP1 gene transcript greatly increases in females but is nearly totally absent in males (13,14). Our objective of *in vitro* transcription studies is to detect the *trans*-acting factors that might

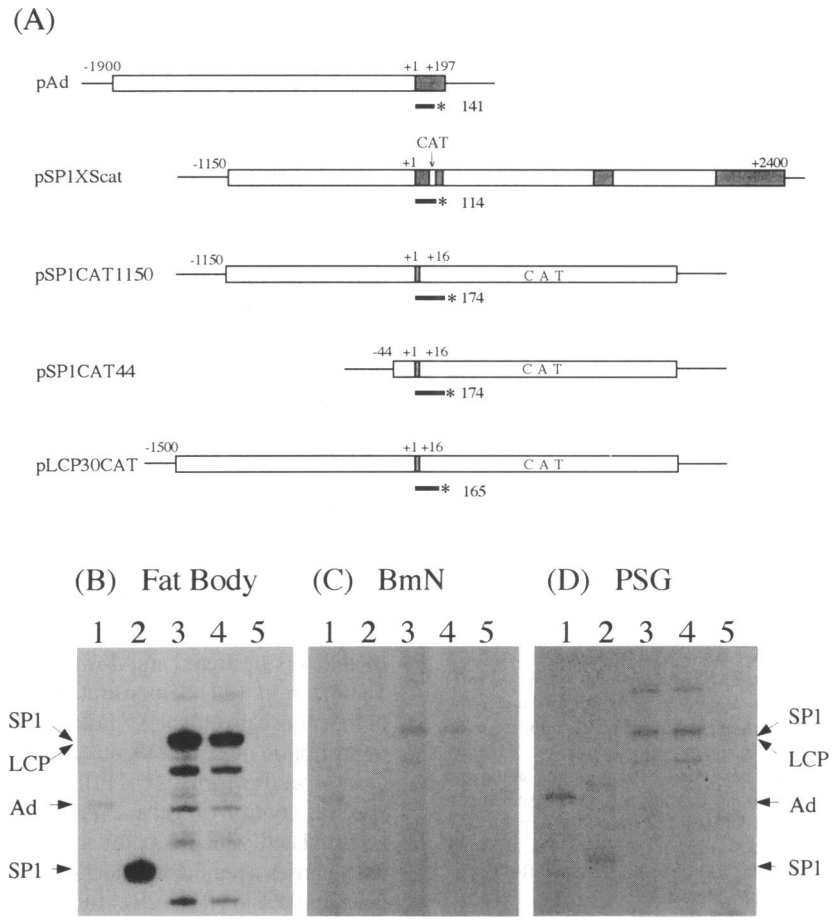

Figure 3. *In vitro* transcription of tissue-specific genes in the fat body nuclear extract. (**A**) Structures of template DNAs and reverse transcripts are illustrated in the same manner as described in Figure 1. Solid bars with asterisks indicate the primer-extended fragments. Numbers to the right indicate the expected sizes of fragments. +1 indicates transcription initiation sites. (**B**) Transcription reactions were performed with 0.6 nM of pAd (lane 1), pSP1XScat (lane 2), pSP1CAT1150 (lane 3), pSP1CAT44 (lane 4) or pLCP30CAT (lane 5) in the fat body nuclear extract (100 µg protein) prepared from the female fifth instar larvae. (**C**) Transcription reactions were performed in the same manner as in (B) except that the fat body nuclear extracts were replaced with the BmN whole cell extract (150 µg protein). (**D**) Transcription was performed as in (B) but with 40 µg protein of PSG extract in place of the fat body nuclear extract. RNA synthesized was analyzed by primer extension assay. Arrows with numbers to the right of plate indicate the sizes in bases of the specific reverse transcripts.

participate in sex-, developmental stage- and tissue-specificity of the *B.mori* gene transcription. To examine whether the fat body *in vitro* system would reflect *in vivo* situation of gene regulation, transcription of the SP1 gene templates was studied employing the nuclear extracts prepared from the homologous fat body and those from the heterologous cultured BmN cells and PSG. In this study, however, transcription reaction was carried out in the presence of larger amounts of nuclear proteins (100 µg/reaction) and much lower concentrations of templates (0.6 nM/reaction) than those in the experiment shown in Figure 1 so as to bring the reaction condition closer to the *in vivo* situation, but even then the template concentrations employed were about 100 times as much as that estimated in the fat body nuclei.

When transcription reaction was performed in the fat body nuclear extract prepared from the fifth instar female larvae, the plasmid clones containing various lengths of the SP1 gene sequences gave products with expected sizes. As is evident from Figure 3B, transcription of the SP1 gene constructs was much more prominent than that of pAd and the larval cuticle protein gene construct (LCP30CAT), both of which were heterologous templates. The plasmid pSP1XScat contained SP1 gene sequence including normal lengths of 1st and 2nd exons except that a portion of 1st exon sequence was replaced by the CAT structural gene sequence. Efficient transcription of pSP1XScat as well as the 3′ deleted template (pSP1CAT1150) in the fat body system indicates that the 3′-flanking region of transcription initiation site has little effect on *in vitro* transcription (Fig. 3B, lanes 2 and 3). Similarly, transcriptional efficiency was not impaired by the removal of 5′-flanking sequence spanning from nt positions –1150 to –44 of the SP1 gene (Fig 3B, lane 4).

In contrast to the reaction in the fat body systems, the transcription rates of SP1 gene constructs were much reduced in the nuclear extracts prepared from BmN cells and PSG (Fig. 3C and D). In these heterologous systems, pAd and SP1 gene constructs were transcribed with nearly equal but low efficiency at the template concentrations employed. Thus, *in vitro* transcription from SP1 promoters in the fat body cell-free system can reproduce, at least in part, the *in vivo* processes. These results strongly suggest that specific factors in the fat body extracts participate in the tissue-dependent transcription of the SP1 gene.

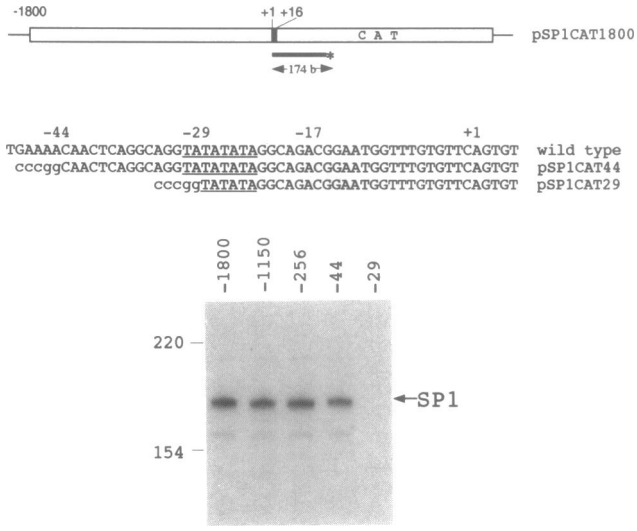

Figure 4. Transcription of the 5′ deleted SP1 gene. The 5′ deleted pSP1CAT (0.6 nM) was transcribed *in vitro* in a mixture containing 100 μg protein of the fat body nuclear extract. The transcripts were analyzed by primer extension as described in Figure 3 legend. The uppermost diagram shows pSP1CAT1800 template. Shaded area represents exon. Bold line with asterisk under the diagram indicates the ^{32}P-labelled 174 base fragment which is supposed to be reverse transcribed from the *in vitro* transcript by primer extension. Structure of SP1CAT series templates are shown above the radioautogram. Capital and lower case letters represent the 5′ flanking regions and vector sequences, respectively. Underline indicates the TATA box. The transcript of pSP1CAT series plasmids is indicated by an arrow to the right of radioautogram. Numbers to the left of the radioautogram represent sizes of end-labeled *Hin*fI-digested pBR322 which was co-electrophoresed as size marker.

Transcription of the 5′ deleted SP1 gene

We previously showed that the sequence between nt positions –44 and –29 of the SP1 gene including the first TA of TATA box sequence is indispensable and sufficient for efficient transcription in a cell-free system derived from the cultured BmN cells (16). To define the promoter proximal region responsible for the efficient transcription of SP1 gene in the homologous fat body nuclear extract, a series of 5′ deleted mutants of SP1 gene were prepared and transcribed in the fat body nuclear extract from the female fifth instar larvae. Figure 4 shows the results of primer extension analyses of the *in vitro* transcripts directed by the mutated SP1 gene promoters. In agreement with the result shown in Figure 3, no substantial increase or decrease in the transcription rate is seen with any of the mutants or the wild type template unless the deletion extends to nt position –29 in the TATA box sequence. Taken together, from the results shown in Figures 3 and 4, it can be concluded that the core promoter region of SP1 gene appears to be sufficient for sustaining transcription reaction also in the fat body cell-free system.

DISCUSSION

In the present study, we have developed an efficient *in vitro* transcription system from the larval fat body of *B.mori*. Although cell-free systems for the transcription of insect genes have been developed from tissues or cell lines of several insect species (1,7,16,21,22), the system derived from the fat body cells has never been established. As far as we know, this is the first cell-free system developed from the insect fat bodies.

We demonstrated that the cell-free extracts prepared from fat body cells are more effective than those from the cultured BmN cells and posterior silk glands in transcribing the gene constructs bearing the core promoter sequence of the SP1 gene which is actively transcribed in the larval fat body of *B.mori*. Heterologous templates that include AdML gene and a larval cuticle protein gene of *B.mori* were only poorly transcribed at low template concentrations in the fat body system. The results indicate that the *in vitro* system we developed is sufficient for faithfully reproducing *in vivo* processes bringing about tissue-specific expression of plasma protein genes. We suggest that specific factor(s) in the fat body nuclear extract participates in the tissue-dependent transcription of plasma protein genes. Experiments employing a series of 5′-deleted SP1 genes indicated that the tissue-specific activating sequence(s) is located in the region proximal to and surrounding the TATA box of SP1 gene.

Recent studies have indicated that core promoters of several eukaryotic genes have inherent function for tissue-specific transcription. The core promoter (–37 to +10) of fibroin gene governs the PSG specific transcription which is further enhanced by the modulators upstream and downstream of the core promoter (23). Tamura *et al.* (24) demonstrated that core promoter (–36 to +12) of the mouse myelin basic protein gene regulates the brain-specific transcription in a homologous cell-free system, and suggested that tissue-specific forms of TFIID may be involved in the tissue-specific control of gene expression. Furthermore, reports have accumulated which support a possibility that the core promoter itself carries a regulatory function and determines specificity of the gene expression (25–29). In view of these observations, it is probable that the core promoter region of SP1 gene plays a role in the fat body-specific gene expression *in vivo*. Obviously, it is highly likely that, in addition to the core promoter, enhancer sequence(s) located away from the promoter region also participates in the regulated expression of SP1 gene. Unfortunately, however, it would be difficult in cell-free systems, as in the present study, to reproduce the functions of enhancer elements located distant from the transcription initiation site (30). Nevertheless, the fat body cell-free system is advantageous for studying regulatory mechanisms underlying the tissue-dependent transcription of plasma protein genes. This system is satisfactory for assembling basic transcription machinery on the promoters of exogenously added templates and to initiate accurate gene transcription. Sizable quantities of fat bodies at defined developmental stage and sex can be obtained with little difficulty, providing the source is particularly suited for preparing large quantities of nuclear extract for the isolation of transcription factors. Experiments are in progress to isolate the fat body nuclear factor(s) responsible for the SP1 gene-specific enhancement of transcription *in vitro*.

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