Volume 14 Number 6 1986 Nucleic Acids Research

Homologies of nucleotide sequences in the 5'-end regions of two developmentally regulated genes of *Sarcophaga peregrina*

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Received 31 December 1985; Revised and Accepted 20 February 1986

ABSTRACT

In the previous paper we demonstrated that the storage protein gene and 25-kDa protein gene are expressed sequentially in the fat body of middle third-instar Sarcophaga peregrina larvae. In this paper, we showed that the expressions of these two genes are regulated at the transcriptional level, and searched for homologous nucleotide sequences in the two genes in the vicinity of their 5'-ends, assuming that these two genes are regulated by a common mechanism. We selected 9 homologous sequences and found that most of them were distributed in two clusters located between positions -400 and +1. We identified an SV40 enhancer core segment-like sequence in the nontranslated region of the first exons of both genes, which might explain the efficient transcriptions of these two genes.

INTRODUCTION

The mechanism by which selective gene transcription is controlled is one of the most important problems to be solved. The interaction of specific protein factors with distinct DNA sequences or the higher-order structure of certain genes is thought to be essential for preferential transcription of these genes (1), and evidence that supports this idea has been accumulating (2-5). One way to approach this problem is to look for a common structure in multiple genes that are expressed coordinately, because this structure could be a signal for specific binding of common regulatory molecules that allow these genes to be transcribed (6).

We previously described molecular cloning and expression of two genes encoding the storage protein and 25-kDa protein of the flesh fly Sarcophaga peregrina (7-10). These genes are specific in the following two points. First, mRNAs for these genes are quite abundant, and when poly(A)$^+$ RNA from the fat body of third

instar larvae is translated in vitro, the major products are storage protein and 25-kDa protein (8). Second, the expressions of these two genes are strictly restricted to a specific developmental stage of larvae. Namely, there is only a short interval between the sequential expressions of these two genes in the fat body of middle third-instar larvae (8). The similarity in their expressions in terms of abundancy of mRNA, the stage of expression and its tissue specificity strongly suggested that their expressions are under similar control. To clarify the regulation of the expression of these two genes, we investigated the change of transcription rate of these two genes in the third instar in comparison with the accumulations of the two mRNAs. We also determined the cap site of the storage protein gene and analyzed the nucleotide sequences around the cap sites of the storage protein and 25-kDa protein genes to see if the two genes contain common sequences. Results suggested that the expressions of these two genes are at least partly controlled by a common factor(s).

MATERIALS AND METHODS

Recombinant DNA

The plasmids used in this study were pS25 and pSP2. pS25 is a subclone of the 2.3 kbp EcoRI-HindIII fragment of a genomic DNA clone of the 25-kDa protein gene inserted into pUC8 and contains the entire coding sequence for 25-kDa protein (10). pSP2 is a subclone of the 2.1 kbp HindIII-HindIII fragment of a genomic DNA clone of storage protein gene λSP19 inserted into pBR322 (9). As shown in the text, this clone contained the cap site and a part of the coding sequence of the storage protein. Propagation of bacteria and preparation of plasmid DNA were done by the standard procedures described by Maniatis et al. (11). Purified DNA was labeled with ^{32}P by nick-translation (12) at a specific activity of about 1×10^8 cpm per μg DNA when used as a probe for hybridization.

Enzymes

Restriction enzymes and polynucleotide kinase were purchased from Takara Shuzo Co. Reverse transcriptase and S1 nuclease were obtained from Bio-Rad and Sigma, respectively.

Experimental animals and isolation of fat body

Larvae of the flesh-fly Sarcophaga peregrina were reared by the method of Ohtaki (13). The physiological age of larvae was easily controlled by dry-wet treatment. Third instar larvae kept in plastic containers do not pupate because moulting hormone is not secreted from the ring gland, but when they are transferred to dry conditions, they start to pupate after 16 h at 27°C. Ecdysis to the third instar is completed about 35 h after hatching, so fat body samples were collected from larvae kept under wet conditions for various times after this stage. To obtain pupal fat body, we transferred larvae to dry conditions 110 h after hatching. Exactly 16 h later, most larvae started to pupate. Fat body samples were collected with time after puparium formation. Fat bodies were excised under a binocular microscope, washed with insect saline (130 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$), and stored at -80°C before use.

In vitro transcription in isolated nuclei

In vitro transcription in isolated nuclei was performed by a modification of the methods of Natori (14), and Nakanishi and Garen (15). Frozen fat bodies from 5-10 larvae were thawed in 3 ml of ice-cold buffer-I (15 mM Hepes, pH 7.5, 15 mM NaCl, 60 mM KCl, 15 mM 2-mercaptoethanol, 0.5 mM spermidine, 0.15 mM spermin, 0.34 M sucrose, 2 mM EDTA, 0.5 mM EGTA, and 0.5 mM PMSF), and were disrupted by 8 strokes of a glass/teflon homogenizer. The homogenate was centrifuged at 1,000 x g for 10 min at 4°C, and the pellet was suspended in 5 ml of buffer-II, which had the same composition as buffer-I except that the concentrations of EDTA and EGTA were decreased to 0.1 mM. The suspension was centrifuged at 1,000 x g for 10 min at 4°C and the pellet was suspended in 500 µl of buffer-III (50 mM Hepes, pH 7.5, 11 mM NaCl, 75 mM KCl, 11 mM 2-mercaptoethanol, 0.36 mM spermidine, 0.11 mM spermin, 0.24 M sucrose, 0.07 mM EDTA, 0.07 mM EGTA, 0.36 mM PMSF, 5 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 0.25 mM ATP, 0.25 mM GTP, 0.25 mM CTP, and 0.025 mM UTP). The suspension was then transferred to a tube containing 100 µCi of [α-^{32}P]UTP (410 Ci/mmol, Amersham Japan Corp.) and transcription was carried out at 25°C for 20 min. The reaction was stopped by addition of 13

µl of 20% SDS and 20 µl of 200 mM EDTA solution. RNA was then extracted, precipitated with ethanol, and dissolved in hybridization buffer consisting of 20 mM sodium phosphate, pH 6.5, 5 x SSC (1 x SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone, 100 mg/ml of denatured salmon sperm DNA, 0.1% SDS, and 50% formamide. This ^{32}P-labeled RNA synthesized in isolated nuclei (6.5 x 10^4 cpm) was used for hybridization with 2 µg of pS25, pSP2 or pBR322 immobilized on a nitrocellulose paper. Hybridization was performed at 42°C for 44 h and the nitrocellulose paper was washed twice with 2 x SSC containing 0.1% SDS, and three times with 0.1 x SSC containing 0.1% SDS at room temperature. The paper was then dried and exposed to Kodak XAR-5 X-ray film with an intensifying screen at -80°C.

Northern blot analysis of RNA

Preparation of total RNA and Northern blot analysis were performed by the methods of Tahara et al. (9) and Thomas (16), respectively. The final RNA precipitate was dissolved in distilled water and stored at -20°C. Samples of 10 µg of RNA were subjected to electrophoresis in 1.2% agarose gel containing 2.2 M formaldehyde, 20 mM morpholinopropanesulfonic acid, 1 mM EDTA, and 5 mM sodium acetate, pH 7.0. The gel was stained with 0.4% ethydium bromide to locate marker RNA, and then blotted onto nitrocellulose paper. Hybridization was done with nick-translated probes at 42°C for 13 h in the same hybridization buffer as used for analysis of in vitro transcripts. The nitrocellulose paper was then washed twice with 2 x SSC containing 0.1% SDS and three times with 0.1 x SSC containing 0.1% SDS at room temperature, and subjected to autoradiography.

S1 nuclease assay and primer extension assay

S1 nuclease assay and primer extension assay were performed by modifications of the methods of Berk and Sharp (17), and McKnight and Kingsbury (18), respectively, as described previously (10).

RESULTS

In vitro transcriptions of the storage protein and 25-kDa protein gene in isolated nuclei

We previously reported a marked increase in the levels of accumulated mRNAs of the genes encoding the storage protein and 25-kDa protein in the fat body of larvae of Sarcophaga peregrina in the middle of the third instar (8). These two genes are good models of developmentally regulated genes and are useful for studies on regulation of transcriptions, because their mRNAs are very abundant (8). However, several examples have been reported of selective accumulations of certain gene products that are not due to increased transcription but to increased stability of the mRNA (19-23). Therefore, it is important to determine the reason for the apparent increases in the amounts of storage protein and 25-kDa protein mRNA. For this, we determined the levels of transcription of the two genes in isolated nuclei prepared from fat bodies collected with time after ecdysis to the third instar.

Nuclei were isolated from fat bodies collected at 15 different stages from the early third-instar to early pupal stage, and the transcription reaction, which had been initiated in vivo, was continued in vitro in reaction mixture supplemented with substrates including [α-^{32}P]UTP and other essential components. The level of transcription was determined by hybridization of the ^{32}P-labeled RNA thus synthesized with cloned DNA of the storage protein gene and 25-kDa protein gene, respectively, which had been immobilized on a nitrocellulose paper. On the other hand, total cellular RNA was prepared from the same samples of fat body and the levels of accumulated mRNA of the two genes were analyzed. We could, therefore, compare the levels of transcription and accumulated mRNA directly. As shown in Fig. 1, 3.4 kb mRNA for the storage protein appeared at 39 h, reached a maximum level at 46 h after hatching, and persisted until 75 h after hatching, whereas 1.0 kb mRNA for the 25-kDa protein appeared at 57 h and reached a maximum level at 75 h after hatching. Thus, these two genes were activated and expressed in the fat body of third instar larvae in almost the same way with an interval of about 18 h between their

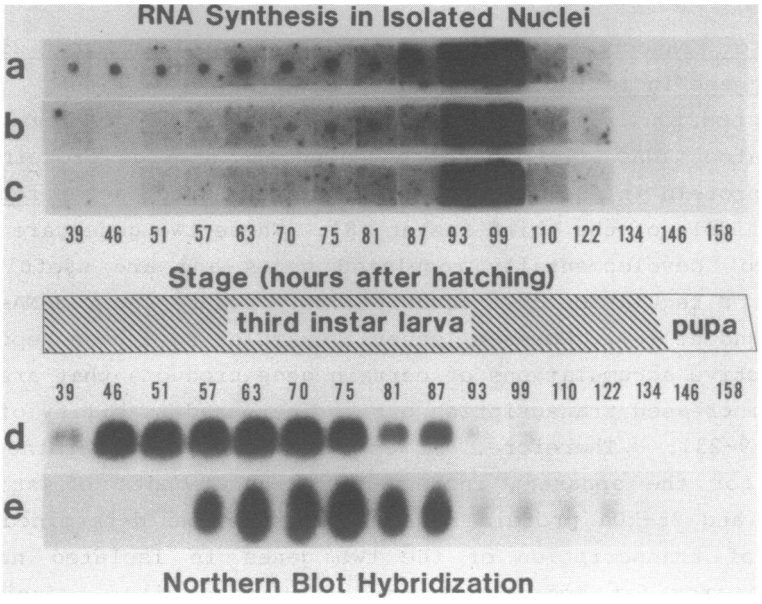

Fig. 1 Change in the levels of accumulated mRNA and transcription of the storage protein gene and 25-kDa protein gene. Northern blot analysis was done with total cellular RNA extracted from fat bodies of animals staged at the various times indicated. (d) and (e) are for analyses of storage protein mRNA and 25-kDa protein mRNA, respectively. Change in the transcriptional level was determined by in vitro transcription in isolated nuclei prepared from fat bodies collected at each stage. (a), (b) and (c) are analyses with nitrocellulose papers containing storage protein gene, 25-kDa protein gene and pBR322, respectively. Autoradiographed films of nitrocellulose papers after hybridizations are shown.

expressions. Dot-blot analyses of the levels of transcription of these two genes in nuclei isolated from the same fat body samples are shown in Fig. 1. The transcriptional levels of the two genes changed in accordance with change in the levels of accumulated mRNA. Intense signals dispersed all area of autoradiographs of 93 h and 99 h are thought to be a background caused by unknown reasons, since all three samples of these stages exhibited almost the same pattern. These results clearly indicate that the accumulations of mRNAs for the storage protein and 25-kDa protein are mainly due to activations of transcription of these protein genes, not to stabilization of

their mRNAs, which are synthesized continuously. Since the times of inductions of transcription of these two genes are very similar, differing by about 18 h, and since these transcriptions occurred in the same tissue, and these two genes were transcribed much more extensively than other genes, these two genes may operate, at least partly, by a common regulatory mechanism. We, therefore, tried to analyze the nucleotide sequences around the transcription initiation sites and 5'-flanking regions of the two genes to determine whether similar sequences are present in these regions that could serve as recognition signals for a tissue and stage specific transcription factor(s).

Determination of the transcription initiation site of the storage protein gene

For comparison of the primary structures of the putative regulatory regions of the two genes, the transcription initiation site (+1) of the genes must be determined to align the genes. That of the 25-kDa protein gene has been mapped (10). Here we analyzed that of the storage protein gene by S1 nuclease assay and primer extension assay.

S1 nuclease assay was performed with 5'-end-labeled 0.83 kbp HindIII/BamHI fragment indicated in Fig. 3(B) as a probe. This probe was chosen on the basis of the results of a rough estimation of +1 by hybridization of DNA fragments obtained by restriction enzyme digestion of cloned DNA to mRNA immobilized on nitrocellulose paper (data not shown). The results are shown in Fig. 2(A) with Maxam-Gilbert ladders (24) of intact probe as molecular size markers. As is evident from lane 2, two distinct and three faint protected fragments with closely similar sizes were detected, and the end points of each fragment on cloned DNA are shown with dots on nucleotide sequences at the right of the figure.

On the other hand, a primer extension experiment was performed with an 18-base oligonucleotide synthesized according to the nucleotide sequences of the storage protein gene inidicated in Fig. 3(A) (at positions +11 to +28) as a primer. As shown in Fig. 2(B), only one band was obtained in a primer extension experiment and the site of transcription initiation

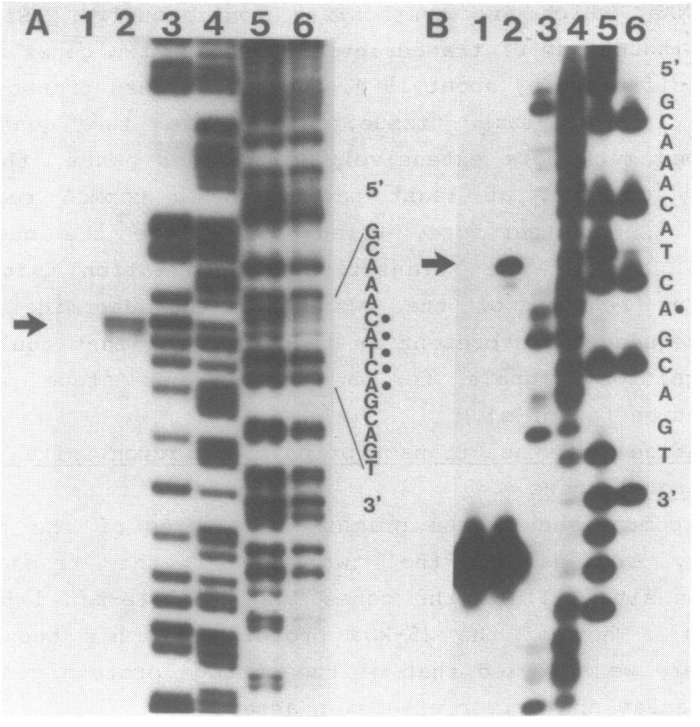

Fig. 2 Determination of the transcription initiation site (+1) of the storage protein gene. (A) S1 nuclease assay. Samples of 200 µg of total cellular RNA prepared from whole larvae at 65 h after hatching (lane 2) or E. coli tRNA (lane 1) were hybridized to the probe and treated with S1 nuclease, and protected DNA fragments were analyzed by 8 M urea-8% acrylamide gel electrophoresis followed by autoradiography. The sizes of protected DNA fragments were determined with molecular size markers of Maxam-Gilbert sequence ladders of intact probe (lane 3, G; lane 4, G+A; lane 5, C+T; lane 6, C). (B) Primer extension assay. Samples of 20 µg of total cellular RNA prepared from whole larvae at 65 h after hatching (lane 2) or E. coli tRNA (lane 1) were hybridized to 5'-end-labeled primer and cDNA was synthesized with reverse transcriptase. The products were analyzed by 8 M urea-20% acrylamide gel electrophoresis followed by autoradiography. The size of the extended product was determined with sequence ladders of Sau3A-TaqI fragment (see Fig. 3(B)), (lane 3, C; lane 4, C+T; lane 5, G+A; lane 6, G). Positions of resulting DNA fragments in each experiment are indicated by arrows and end points of each fragment on storage protein gene are shown with dots on nucleotide sequences of coding strand at the right.

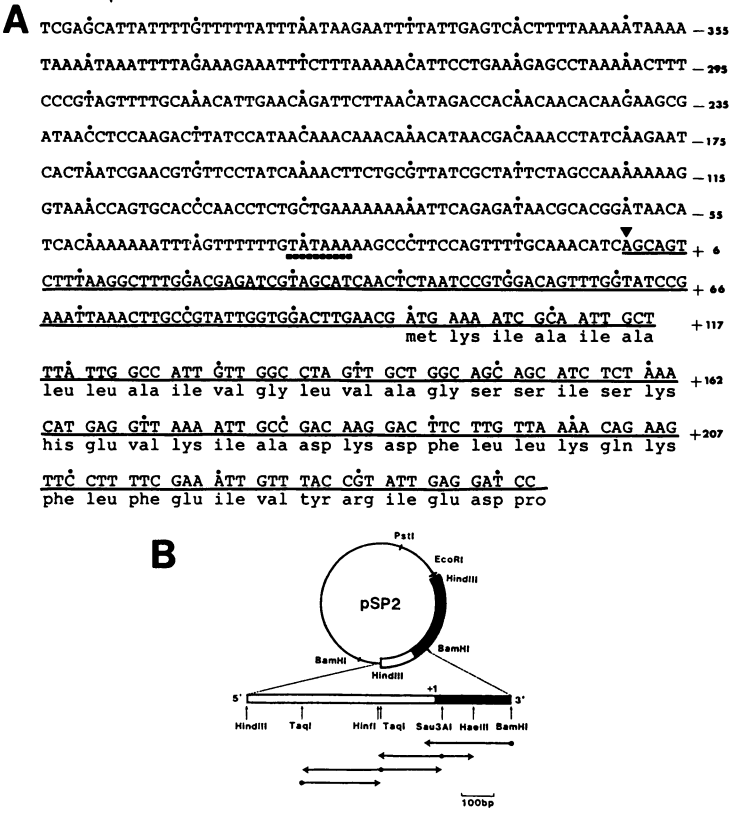

Fig. 3 Nucleotide sequence of the 5′-coding and flanking region of the storage protein gene. (A) Nucleotide sequence of the 5′-flanking region and part of the coding region of the storage protein gene. Positions were numbered relative to the site of transcription initiation (▼). A region of presumed mature mRNA is shown with an under line. The amino acid sequence of the storage protein deduced from the nucleotide sequence is shown. A sequence corresponding to the "TATA box" is indicated by a broken under line. (B) The strategy of sequencing by the method of Maxam and Gilbert is shown. DNA fragments obtained by restriction enzyme digestions were labeled with ^{32}P at their 5′-ends by polynucleotide kinase and used for chemical cleavage.

deduced from this result coincided with one of the sites determined by S1 nuclease assay (Fig. 2(A)). We, therefore, presumed that the site of transcription initiation of the storage protein gene is A indicated by a closed triangle in Fig. 3(A).

Table 1. Sequence Homology between Storage Protein gene and 25-kDa Protein Gene

	DNA Sequence	Storage Protein Gene 25-kDa Protein Gene	Homology(%)
1	TATTTAATAAG-AATTTTAT [-395,-377] :: ::::::: ::: :::: TAATTAATAATTAATATTAT [-308,-289]		80
2	TTTAAAAATAAAATAAAATAA [-367,-347] :::::: :::: :: ::::: TTTAAAC-TAAATTAGAATAA [-256,-237]		81
3	TTAAAAATAAAATAAAATA-AAT [-366,-345] ::::: :::: :::: :: ::: TTAAATTTAAACTAAATTAGAAT [-261,-239]		78
4	AAATTTTAGAAAGAAATTTCTT [-348,-327] ::::::: : ::::::: :: AAATTTTTATATGAAATTTGTT [-287,-266]		77
5	GAAAAAAAAAA [-88,-79] :::::::::: GAAAAAAAAAA [-85,-76]		100
6	AAAAAA-AAATTCAGAGATAACG [-87,-66] :::::: ::: ::: : :::::: AAAAAATAAAATCA-A-ATAACG [-59,-39]		83
7	AACATCA-CAAAAAA [-58,-45] :::: :: ::::::: AACAGCAGCAAAAAA [-68,-54]		87
8	CAAACATCAGCA [-8,+45] :::::: ::::: CAAACAGCAGCA [-70,-59]		92
9	TGGTGGACTTGAACGATGAAAAT [+85,+107] :: :::: : ::: :: ::::: TGATGGATTGGAATAATAAAAAT [+87,+109]		74

Nucleotide positions of 5′ and 3′ boundary are indicated in brackets. Matched nucleotides between two sequences are indicated with dots. Numbers at the left of sequences correspond to those in Fig. 4.

Comparison of nucleotide sequences in the vicinity of the transcription initiation sites of the storage protein gene and 25-kDa protein gene

The nucleotide sequence of a genomic DNA clone for the storage protein gene (pSP2) containing the 5′-end and flanking region was determined by the method of Maxam and Gilbert (24) by

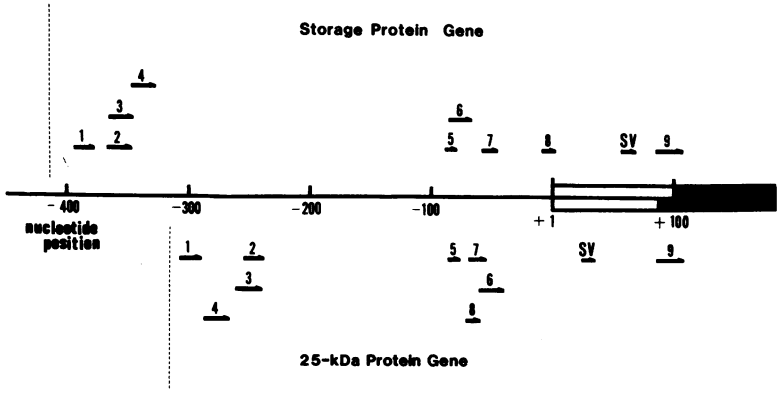

Fig. 4 Localizations of homologous sequences in the storage protein gene and 25-kDa protein gene. Regions of the two genes containing homologous sequences that are localized within 100 bp when the transcription initiation sites of the two genes are aligned are shown with numbered arrows. Regions that contain sequences homologous to the core segment of SV40 enhancer are indicated by arrows with the letter SV. Each arrow-head indicates its direction. Locations of homologous sequences in the storage protein gene are shown at the top part and those in the 25-kDa protein gene are shown at the bottom part of the figure. Open bar and closed bar show nontranslated and translated regions, respectively.

the strategy shown in Fig. 3(B). A total sequence of 656 bp was determined and the TATAAA sequence as a typical "TATA box" (25) was found at positions -31 to -26. This shows that the site of transcription initiation determined by S1 nuclease assay and primer extension assay is correct. No sequence corresponding to a "CAAT box" (26) was found in the 5′-flanking region of the storage protein gene, which has been found to be present at about -90 in the 25-kDa protein gene (10). A putative initiation codon is located at positions +100 to +102, assuming that it belongs to the first exon. This was followed by an open reading frame. We could deduce the 47 amino acid residues encoded by this frame.

We next examined if there is any structural similarity between the storage protein gene and 25-kDa protein gene in their 5′-flanking and transcription initiation regions. Analyses were done by computer with the program developed by Kanehisa (27) with a maximum probability of 2.1×10^{-5}. We

selected homologous sequences in the two genes. Our criterion for selection of these sequences was that two sequences should be close enough to be localized within 100 bases when the transcription initiatin sites of the two genes were aligned. In this way, we finally picked up 9 regions, which are shown in Table 1. Their positions relative to the transcription initiation site (+1) are indicated in Fig. 4. Two clusters of homologous sequences were found. Namely, sequences 1 to 4, and sequences 5 to 8 are localized between positions -400 and -200, and between positions -100 and +1, respectively. Some of these sequences may be involved in the common mechanism regulating the expressions of these two genes. Another homologous sequence found in the nontranslated region of the first exons of both genes (designated as region SV in Fig. 4) has extensive homology with the core segment of SV40 enhancer, GGTGTGGAAAG (28). A sequence GGTATCCGAAAT located at positions +59 to +70 of the storage protein gene has 67% homology with the core segment of the SV40 enhancer, and a sequence GGTGTTGTAAAC located at positions +26 to +37 of the 25-kDa protein gene has 75% homology with it.

DISCUSSION

In this work, we demonstrated that stage specific expression of two developmentally regulated genes of Sarcophaga peregrina, the storage protein gene and 25-kDa protein gene, is due to gene activation at the transcriptional level. Since these two genes are expressed sequentially in the fat body of third instar larvae with only a short interval between their expressions (8), and since the products of these two genes are much more abundant than those of other genes (8), they are good models for use in studies on sequential expressions of tissue specific genes. As a first step in analysis of the mechanism of coordinated expression of these two genes, we compared the nucleotide sequences of the 5′-end and flanking regions of the two genes. As described in the text, we found clusters of sequences showing extensive homology in these regions.

There are several reports of gene-specific transcription factors (2-5, 31,32). Therefore, it is possible that only some

of these homologous sequences are common regulatory regions and the expressions of these two genes are controlled by a common factor(s) that interacts with these regions. Then, why is there an interval of 18 h between the expressions of the two genes? We assume that this is not directly related to the homologous sequences in the regulatory regions, but to the overall structure of the chromatin. Once this structure becomes in the transcribable state, each gene may be expressed with the aid of a common transcription factor(s). In fact, we showed that a DNase I hypersensitive site (29) appears at about -300 of the 25-kDa protein gene prior to and during active transcription, and persists until transcription is shut off (30).

We found sequences similar to the core segment of SV40 enhancer (28) in the nontranslated region of the first exons of the two genes It is not certain whether these sequences actually function as enhancers. However, since the efficiencies of transcription of these two genes are extremely high (8), the enhancer-like sequences may modulate their transcription.

Sequence analysis demonstrated that the 5′-end and flanking regions of the storage protein gene and 25-kDa protein gene contain extensive homologous sequences. Therefore, the next step will be to examine whether these homologous sequences function as binding sites of a common transcription factor(s). For this, it may be useful to establish an *in vitro* transcription system for these two genes using a fat body extract of *Sarcophaga* larvae. It may be possible to identify a common transcription factor(s) for the storage protein gene and 25-kDa protein gene that recognizes the homologous sequences found in this study.

ACKNOWLEDGEMENTS

We thank T. Tahara for his contribution in determination of the nucleotide sequence of pSP2. We are also grateful to H. Satow and S. Takada for help in computer analysis, and M. Obinata for valuable discussion. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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