

SEQUENCES OF TWO cDNAs AND EXPRESSION OF THE GENES ENCODING METHIONINE-RICH STORAGE PROTEINS OF *MANDUCA SEXTA**

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Abstract—In *Manduca sexta*, storage proteins accumulate during the final larval stadium for utilization during subsequent larval-pupal-adult transformations. Two cDNA clones (designated clone 119 and clone 201), that represent two distinct but related genes (42% sequence identity), were isolated from a cDNA library prepared from day 7 fifth instar larval fat body and found to encode two different storage proteins synthesized during the last larval instar. Northern blot analyses revealed that the two clones hybridize to 2.4 kb transcripts that are translated to 79 kDa protein products during *in vitro* translation experiments. Clone 119 encodes a methionine-rich storage protein, designated as SP1A, that shares 37% sequence identity with the *Bombyx mori* sex-specific storage protein SP1. Clone 201, on the other hand encodes a storage protein, designated as SP1B, that is more closely related to *B. mori* SP1 (63% identity), and is probably identical to the *Manduca* female-specific storage protein (FSP). Insert DNA from clone 201, but not clone 119, cross-hybridizes to that of FSP cDNA (Webb and Riddiford, *Dev. Biol.* 130, 671-691, 1988a). Both storage proteins are synthesized only in the fat body and only during the fifth larval stadium, indicating tissue- and stage-specific expression of the two genes. Both genes exhibit sex-specific differences in expression. In the fifth larval stadium, the mRNAs for the SP1A and SP1B proteins begin to accumulate at about day 2 in the female fat body but appear 2 or 3 days later in the male fat body. In both sexes SP1A mRNA remains relatively high beyond the time when SP1B mRNA has already declined to low levels, suggesting differences in mRNA stability or expression. Injection of 20-hydroxyecdysone into ligated fifth instar abdomens causes substantial increases in the levels of both mRNAs, whereas topical application of the juvenile hormone mimic, fenoxycarb, to feeding fifth instar larvae produces substantial declines in the mRNA levels, indicating hormonal effects at the transcriptional level. The data support the hypothesis that the expression of these *M. sexta* methionine-rich storage protein genes is stimulated by ecdysteroid and inhibited by juvenile hormone.

Key Word Index: storage protein; *Manduca sexta*; fat body; cDNA; 20-hydroxyecdysone; juvenile hormone; developmental regulation; tobacco hornworm; fenoxycarb

INTRODUCTION

Storage proteins are useful for studying gene regulation during insect development because their genes are expressed at high levels primarily during the larval stage (Law and Wells, 1989). They are synthesized by the larval fat body, released into the hemolymph, and selectively sequestered from hemolymph by the fat body at a later time (Levenbook,

1985). Although the physiological function(s) of storage proteins is not well elucidated, they may serve as amino acid stores required during nonfeeding periods of insect development.

During the fifth larval stadium of *Bombyx mori* and *Manduca sexta*, two types of storage proteins accumulate in hemolymph; one is called female-specific storage protein (FSP) or SP1, is relatively rich (6-11%) in methionine and is expressed more abundantly in the female than in the male (Tojo *et al.*, 1980; Ryan *et al.*, 1985; Kajiura and Yamashita, 1989), whereas another is called arylphorin or SP2 and is relatively rich (18-25%) in aromatic amino acids (Tojo *et al.*, 1980; Kramer *et al.*, 1980). *M. sexta* FSP is a hexamer composed of 79 kDa subunits that are synthesized in the female 2 days earlier than in the male during the fifth larval stadium, accumulating first in the hemolymph and then in the fat body of both sexes (Riddiford and Hice, 1985; Webb and Riddiford, 1988a, b). Using a cDNA probe specific for FSP, transcripts for this protein were shown to be synthesized in the fat body of male and female *M. sexta*. No sequence information about FSP cDNA has been presented.

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Abbreviations used: Tris, Tris (hydroxymethyl) amino-methane; EDTA, ethylenediaminetetraacetic acid; FSP, female-specific protein; SP1, storage protein 1; SP2, storage protein 2; SSC, 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7; SDS, sodium dodecyl sulfate; 20-HE, 20-hydroxyecdysone; JH, juvenile hormone; BMSP1, *Bombyx mori* methionine-rich storage protein.

In the course of studies to identify genes for proteins that are critical for molting and are relatively abundant during the larval-pupal-adult transformations of *M. sexta*, we isolated two cDNA clones from a cDNA library prepared using mRNA from day 7 fifth instar larvae. These clones were sequenced to determine their relationship to genes for other proteins that have been described previously in insects. Both of the *M. sexta* cDNA clones code for 79 kDa proteins. Even though the two clones do not cross-hybridize under moderate stringency conditions, the predicted amino acid sequences of their protein products are related to that of the methionine-rich SP1 storage protein of *B. mori* (Sakurai *et al.*, 1988a, b). The absence of cross-hybridization between the two cDNAs has allowed a quantitative analysis of the respective mRNAs in embryos and tissues from *M. sexta* fourth and fifth instar larvae. The two SP1-type genes were found to be transcribed independently of each other in a sex-dependent manner and their expression is up-regulated by ecdysteroid and down-regulated by juvenile hormone.

MATERIALS AND METHODS

Insect rearing, hormone treatments, and tissue collection

M. sexta eggs were supplied by the Biosciences Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota, U.S.A. Larvae were raised on an artificial diet according to Bell and Joachim (1976) at $27 \pm 1^\circ\text{C}$ using a photoperiod of 16 h light and 8 dark. Fourth and fifth instar larvae and embryos were used for all experiments. The larvae were selected on the day of ecdysis (day 0). By the end of day 3, fifth larval instars had attained a weight of 8–10 g. Wandering stage (w), which was identified as the day of both dorsal heart exposure and wandering behavior, occurred on day 4. On day 6, ocellar retraction began when the larval head capsule was slipped, marking the beginning of a large increase in hemolymph 20-hydroxyecdysone titer (Mitsui and Riddiford, 1976). Day 8 was marked by the appearance of the tanned, metathoracic brown bar (BB) of the new pharate pupal cuticle. Fifth instar larvae were sexed between day 0 and day 1 by observing the presence (male) or absence (female) of a genital pore on the ninth abdominal segment (Stewart *et al.*, 1970). Day 2 fifth instar larvae were ligated with thread between the first and second abdominal segments in order to isolate the abdomen from endocrine organs in the head and thorax (Bollenbacher *et al.*, 1975; Riddiford, 1985; Fukamizo and Kramer, 1987). At 48 and 72 h after ligation, the abdomens were injected through a proleg with $7.5 \mu\text{g g}^{-1}$ body weight 20-hydroxyecdysone (Sigma Chemical Co., St Louis, Mo.) dissolved in 10% isopropanol (Fukamizo and Kramer, 1987). Fifth instar larvae were also treated with a juvenile hormone analogue, fenoxycarb (Maag Agrochemical Co., Vero Beach, Fla), by application of $5 \mu\text{g}$ in $1 \mu\text{l}$ ethanol along the dorsal midline at day 2 and day 3. Fifth instar fat body, midgut, integument and muscle and fourth instar fat body were dissected in 0.13 M sodium chloride, 5 mM potassium chloride, and 2 mM calcium chloride containing a few crystals of phenylthiourea to prevent phenoloxidase activity. These tissues were immersed immediately in 4 M guanidine isothiocyanate buffer for RNA extraction (see next section).

RNA isolation

Total RNA was isolated from homogenized tissue and embryonic samples by the method of Chirgwin *et al.* (1979).

Poly(A)⁺ RNA was isolated from total RNA by oligo(dT) column chromatography (Aviv and Leder, 1972). Poly(A)⁺ RNA (1 mg) from day 7 fifth instar fat body was layered on top of a 10–25% sucrose gradient in 10 mM sodium acetate pH 6 with 1 mM EDTA, and centrifuged for 18 h at 38,000 rpm at 4°C in a SW 41 rotor. RNA pellets recovered from 0.5 ml fractions by ethanol precipitation were dissolved in sterile water and translated *in vitro* using a rabbit reticulocyte lysate translation system and L-[³⁵S]methionine (Amersham).

Isolation of genomic DNA

High molecular weight genomic DNA was prepared from day 4 fifth instar larvae by a procedure modified after that of Blin and Stafford (1976). Whole larvae except heads, tails, and gut tissue were cut into small pieces, frozen in liquid nitrogen, and ground to a fine powder using a mortar and pestle cooled in dry ice. The powder was suspended in 10 mM Tris pH 8, 0.1 M sodium chloride, 25 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS) containing $100 \mu\text{g ml}^{-1}$ proteinase K and then shaken for 16–18 h at 50°C . After phenol-chloroform extraction, the aqueous layer was dialyzed against 10 mM Tris, pH 8, 10 mM EDTA overnight. The DNA was banded by two cesium chloride gradient centrifugations using a Beckman VTi 50 rotor at 48,000 rpm and 20°C . Bound ethidium bromide was removed from the DNA by repeated extractions with an equal volume of cesium chloride-saturated butanol. After dialysis overnight against 10 mM Tris pH 8, 10 mM EDTA, the concentration of the DNA was measured spectrophotometrically at 260 and 280 nm.

cDNA library construction

Poly(A)⁺ RNA (40 μg) from a sucrose gradient fraction enriched for fat body mRNA of 1.5–2 kb length capable of coding for 50–90 kDa proteins was used for cDNA synthesis according to Gubler and Hoffman (1983). The double-stranded cDNA product was fractionated through a Sephadex G-50 spin column to remove short cDNA fragments (Maniatis *et al.*, 1982) and then cloned into the Pst I site of pBR322. This cDNA library consisted of approx. 2000 recombinant clones, which were identified by their ability to grow on tetracycline but not on ampicillin plates.

cDNA library screening

Recombinant clones were grown on master plates, transferred onto nitrocellulose filters (BA85, 0.45 μm ; Schleicher and Schuell), and lysed according to Maniatis *et al.* (1982). Filters were hybridized to ³²P-labeled cDNA prepared from day 7 fifth instar RNA under conditions described below for Southern hybridization. The single-stranded [³²P]-labeled cDNA probe was made by reverse transcribing poly(A)⁺ RNA from a sucrose gradient fraction enriched for mRNA encoding 50–90 kDa proteins. 182 clones that exhibited strong positive hybridization to the cDNA probe were further screened by cross hybridization among themselves. To accomplish this, plasmid DNAs were digested to completion with Pst I to release insert DNA and electrophoresed on 1.5% agarose gels (Birnboim and Doly, 1979). Digested DNA was transferred onto a nitrocellulose filter (Southern, 1975) using 20x SSC (SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7) as transfer buffer.

Southern hybridization

Genomic DNA was digested with a 10-fold excess of restriction enzymes Eco RI, Eco RV, and Hind III according to the supplier's instructions (Promega), subjected to electrophoresis, and transferred onto nitrocellulose. The filters were prehybridized, and washed with 0.2x SSC at 65°C as described by Maniatis *et al.* (1982). The ³²P-labeled insert DNA clone 119 and 201 probes had a specific activity of $2\text{--}4 \times 10^8$ cpm μg^{-1} and were used at 1×10^6 cpm ml^{-1} of hybridization solution.

Northern and RNA slot blot hybridization

Total RNA (20 µg) was denatured in formaldehyde and subjected to electrophoresis through a 1% agarose gel containing 2.2 M formaldehyde and blotted onto nitrocellulose according to Thomas (1980).

For slot blot experiments, total RNA (5 µg) was dissolved in 2.2 M formaldehyde with 6x SSC and denatured by heating at 65°C for 15 min. The RNA from all the stages was spotted onto a single nitrocellulose membrane using a minifold II Slot Blot system (Schleicher and Schuell Inc.).

Northern or slot blot filters were probed with a ³²P-labeled cDNA insert (1 × 10⁶ cpm ml⁻¹ hybridization solution) for 12–16 h. The cDNA insert probe had a specific activity of 2–6 × 10⁸ cpm µg⁻¹. Filters were washed at 50°C with the same buffers and conditions employed for Southern blots and exposed to pre-flashed X-ray film.

RNA hybridization signals were quantitated by scanning autoradiograms with an LKB laser densitometer interfaced with an Apple II computer. Autoradiograms representing exposures for different periods of time were analyzed to ensure that all signals were in the linear range. Average values of triplicate or duplicate blots were determined.

DNA sequencing

cDNA inserts were subcloned in either M13mp18 or Bluescript and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using [³⁵S]dATP (Biggin *et al.*, 1983) and Sequenase (Tabor and Richardson, 1987) as prescribed by U.S. Biochemical Corporation. Sequencing reaction products were analyzed on 6% polyacrylamide gels in 7 M urea.

The protein and nucleic acid sequence databases from Protein Information Resource, National Biomedical Research Foundation were searched to identify the clones, using the FASTN and FASTP programs of Lipman and Pearson (1985). Sequences were aligned using the SEQALIGN program kindly provided by Kirk Clark and Gerald Reeck of Kansas State University.

RESULTS

Isolation of cDNA clones for mRNA abundantly expressed in mature larvae

Analysis of approx. 2000 clones from the *M. sexta* cDNA library by colony hybridization detected 182 colonies that hybridized strongly to a cDNA probe prepared from the same enriched RNA fraction used for construction of the library. These colonies were further characterized by hybridization to insert DNA probes prepared from two clones that showed high (clone 119) and medium (clone 201) levels of hybridization to the cDNA. A large percentage of the clones (about 50% hybridized to clone 119 cDNA insert, whereas a smaller percentage (3%) hybridized to clone 201 insert.

Clones 119 and 201 code for 2.4 kb transcripts and 79 kDa proteins

Northern blot analyses of day 6 fifth instar RNA using the two cDNA clones detected approx. 2400 nucleotide long transcripts [Fig. 1(a)]. To determine the size of *M. sexta* proteins corresponding to these RNAs, insert DNAs from the two clones were subcloned into M13 and used to hybrid-select mRNAs which were translated *in vitro*. In both cases, 79 kDa proteins were observed as the major products of the translations after SDS polyacrylamide gel

electrophoresis [Fig. 1(b), lanes 1 and 6]. These results demonstrate that clones 119 and 201 code for mRNAs and proteins of similar size.

Nucleotide sequences of clones 119 and 201

To obtain information about the nature of the genes in *M. sexta* represented by these two clones, sequences of the insert DNAs were determined (Fig. 2). The clone 119 insert is 814 nucleotides long and contains an open reading frame of 750 nucleotides stretching from the 5'-end, capable of coding for 250 amino acids. The insert from clone 201 is 610 nucleotides long and contains an open reading frame of 540 nucleotides stretching from the 5'-end capable of coding for 180 residues of a protein. Thus, the two cDNA clones are incomplete clones and presumably correspond to C-terminal portions of the 79 kDa proteins. Even though the insert DNAs of the two clones do not show cross-hybridization under moderate stringency, computer alignment of the two DNA sequences and the predicted amino acid sequences indicates that the two clones are related. The two clones exhibit 42% DNA and 31% amino acid sequence identity in the alignment shown in Fig. 2.

Clones 119 and 201 code for proteins related to *Bombyx mori* SP1 protein

A search of nucleic acid and protein data bases for related sequences revealed that the proteins encoded by these two *M. sexta* clones are similar to the C-terminal region of *B. mori* sex-specific storage protein SP1 (Izumi *et al.*, 1988; Sakurai *et al.*, 1988a). The protein product predicted from clone 119 exhibits 37% sequence identity with SP1 (Fig. 2). The percentage identity between SP1 and the predicted protein from clone 201 is even greater (63%). It is clear that the SP1-type proteins are indeed related, with 133 positions out of 180 showing partial conservation (at least two sequences identical), and 45 positions showing total conservation. All three sequences are relatively rich in methionine (6–11%), a property that distinguishes SP1 from another storage protein, arylphorin or SP2, which is enriched in aromatic amino acid residues (Ryan *et al.*, 1985). Because of the relatedness of the *M. sexta* proteins to *B. mori* SP1, we designated the proteins encoded by clones 119 and 201 as *M. sexta* SP1A and SP1B, respectively. Two potential glycosylation sites, Asn-X-Ser (Marshall and Neuberger, 1970), are at positions 207–209 and 248–250 in SP1B and SP1A, respectively, in the alignment shown in Fig. 2.

Relationship between *M. sexta* SP1A, SP1B and FSP

Ryan *et al.* (1985) and Webb and Riddiford (1986a) reported that the female-specific protein (FSP) of *M. sexta* was related to *B. mori* SP1. In order to determine whether one of the storage protein cDNAs that we had cloned was related to the FSP gene, we tested for cross-hybridization of insert DNAs from clones 119 and 201 with that of FSP cDNA kindly supplied to us by B. Webb and L. M. Riddiford. As shown in Fig. 3, insert DNA from clone 201 cross-hybridized with the FSP clone insert, whereas that from clone 119 did not hybridize, even under moderate stringency conditions. Thus SP1B is

SP1A	-	R	R	L	N	H	Q	P	F	K	V	T	L	D	V	L	S	D	K	A	V	D	C	V	V	R	25
119	AA	CGC	CGT	CTT	AAC	CAC	CAG	CCA	TTC	AAA	GTC	ACC	CTT	GAT	GTG	CTC	TCC	GAC	AAG	GCT	GTA	GAC	TGT	GTC	GTG	AGG	
201																											
SP1B																											
BMSPI		R	R	L	N	H	H	P	F	Q	V	S	I	D	V	M	S	D	K	T	V	D	A	V	V	R	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
SP1A		I	F	L	G	P	K	E	D	H	L	G	R	L	I	D	I	N	V	N	R	L	N	F	V	E	50
119		ATC	TTC	TTG	GGA	CCG	AGA	GAG	GAC	CAC	CTG	GGC	CGC	CTC	ATC	GAT	ATA	AAC	GTC	AAC	CGC	CTC	AAC	TTC	GTC	GAG	
201																											
SP1B																											
BMSPI		I	F	L	G	P	K	Y	D	C	M	G	R	L	M	S	V	N	D	K	R	L	D	M	F	E	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
SP1A		H	D	T	F	I	Y	K	L	N	T	G	K	N	T	I	V	R	N	S	Y	D	M	H	N	L	75
119		ATG	GAC	ACG	TTC	ATT	TAC	AAA	CTT	AAC	ACC	GGA	AAG	AAC	ACG	ATT	GTG	AGG	AAC	TCC	TAT	GAC	ATG	CAC	AAC	CTT	
201																											
SP1B																											
BMSPI		L	D	S	F	M	Y	K	L	V	N	G	K	N	T	I	V	R	S	S	M	D	M	Q	G	F	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
SP1A		V	R	D	R	I	M	T	R	D	L	W	K	K	V	D	T	I	T	D	M	R	D	M	L	I	100
119		GTC	CGC	GAC	AGA	ATC	ATG	ACA	CCG	GAC	CTC	TGG	AAG	AAG	GTT	GAC	ACC	ATC	ACT	GAC	ATG	AGG	GAT	ATG	CTC	ATA	
201																											
SP1B		I	E	Q	R	P	W	I	R	N	I	W	D	K	T	F	D	N	S	G	S	G	Q	K	T	V	
BMSPI		I	P	E	Y	L	S	T	R	R	V	M	E	S	E	M	M	P	S	G	D	G	Q	T	M	V	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
SP1A		K	D	L	R	-	N	Y	H	T	G	F	P	T	R	L	L	L	P	K	G	F	V	G	G	M	125
119		AAG	GAC	CTA	AGG	---	AAC	TAC	CAC	ACT	GGC	TTC	CCG	ACC	AGG	CTC	CTG	CTA	CCT	AAA	GGT	TTC	GTT	GGT	GGT	ATG	
201																											
SP1B		A	S	W	W	Y	K	T	R	H	G	F	P	H	R	L	L	L	P	L	G	R	Q	G	C	L	
BMSPI		K	D	W	W	C	K	S	R	N	G	F	P	Q	R	L	L	L	P	L	G	T	I	G	C	L	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
SP1A		K	M	M	F	Y	V	I	V	T	P	L	K	-	-	-	L	V	D	N	V	D	M	S	I	L	150
119		AAG	ATG	ATG	TTC	TAC	GTC	ATC	GTG	ACT	CCT	CTG	AAG	---	---	---	TTG	GTA	GAT	AAC	GTT	GAC	ATG	TCT	ATC	CTG	
201																											
SP1B		P	L	Q	L	Y	V	I	V	S	P	V	R	T	G	M	V	L	P	T	I	D	M	N	T	M	
BMSPI		E	M	Q	M	Y	V	I	V	S	P	V	R	T	G	M	V	L	P	T	I	D	M	N	T	M	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
SP1A		D	T	T	R	K	D	L	F	V	D	F	R	S	T	V	L	L	D	K	M	P	L	G	F	P	175
119		GAC	ACA	ACA	CGC	AAG	GAC	CTG	TTC	GTG	GAC	TTC	AGA	TCG	ACT	GTC	TTG	CTG	GAC	AAG	ATG	CCG	CTT	GGT	TTC	CCT	
201																											
SP1B		K	E	R	H	A	C	-	-	-	-	-	R	F	T	V	C	F	D	T	M	P	L	G	F	P	
BMSPI		K	D	R	C	A	C	-	-	-	-	-	R	W	S	C	I	S	T	M	P	L	G	Y	P		
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
SP1A		L	D	R	H	I	D	V	S	T	F	Y	T	P	N	M	K	F	V	D	V	V	I	F	H	K	200
119		CTG	GAC	CGT	CAT	ATT	GAT	GTG	TCG	ACC	TTC	TAC	ACG	CCC	AAC	ATG	AAG	TTC	GTT	GAT	GTG	GTA	ATC	TTC	CAC	AAG	
201																											
SP1B		F	D	R	Q	I	D	M	T	Y	F	F	T	N	M	K	F	T	D	V	M	V	Y	R	K	K	
BMSPI		F	D	R	P	I	D	M	A	S	F	F	T	S	N	M	K	F	A	D	V	M	I	Y	R	K	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
SP1A		K	Q	V	C	D	M	R	T	R	W	D	R	W	V	L	R	N	Y	N	M	V	D	R	T	P	225
119		AAG	CAA	GTG	TGC	GAC	ATG	AGA	ACC	CGA	TGG	GAC	CGC	TGG	GTG	CTG	AGG	AAC	TAC	AAT	ATG	GTG	GAC	CGT	ACG	CCC	
201																											
SP1B		D	L	S	T	M	S	N	T	S	-	-	-	-	-	-	-	K	N	I	D	T	S	N	M	V	
BMSPI		D	L	-	G	M	S	N	T	S	-	-	-	-	-	-	-	K	N	I	D	T	S	N	M	V	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
SP1A		I	N	S	D	T	Y	F	V	D	T	D	V	H	M	N	I	N	T	K	V	D	S	N	V	S	250
119		ATC	AAC	TCA	GAC	ACG	TAT	TTC	GTT	GAC	ACT	GAT	GTT	CAC	ATG	AAC	ATT	AAC	ACT	AAA	ATC	GTC	AGC	AAT	GTC	AGC	
201																											
SP1B		M	K	K	D	D	L	T	Y	L	D	S	D	M	L	M	H	R	T	Y	K	D	V	M	M	M	
BMSPI		M	K	K	D	D	L	T	Y	L	D	S	D	M	L	M	H	R	T	Y	K	D	V	M	M	M	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
SP1A		V	F	D	L	>																					
119		GTG	TTT	GAT	TTG	TAA	ATG	TAA	TCA	GCA	TC	ACTTACTGCAATACTTTGTGACCATGGCGAAAATAGCATCCTAAA															
201																											
SP1B		A	S	D	N	M	L	R	M	>																	
BMSPI		S	M	M	N	>																					
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		

Fig. 2. Nucleotide sequences of insert DNAs from *M. sexta* clones 119 and 201, their predicted amino acid sequences, and alignments of storage proteins from *M. sexta* (SP1A and SP1B) and *B. mori* (BMSPI). The aligned positions of the amino acids are numbered from the amino- to the carboxyl-terminal direction. The sequences were aligned using the SEQALIGN program package of Clark and Reeck (in preparation) with Trace 2 subprogram to determine the optimum number of gaps. + and * indicate identity of 2 and 3 amino acids, respectively, in the position. The sequence of *B. mori* SP1 (BMSPI) shown extends from position 506 to the C-terminus at position 747 (Sakurai *et al.*, 1988a).

(a) 119 201

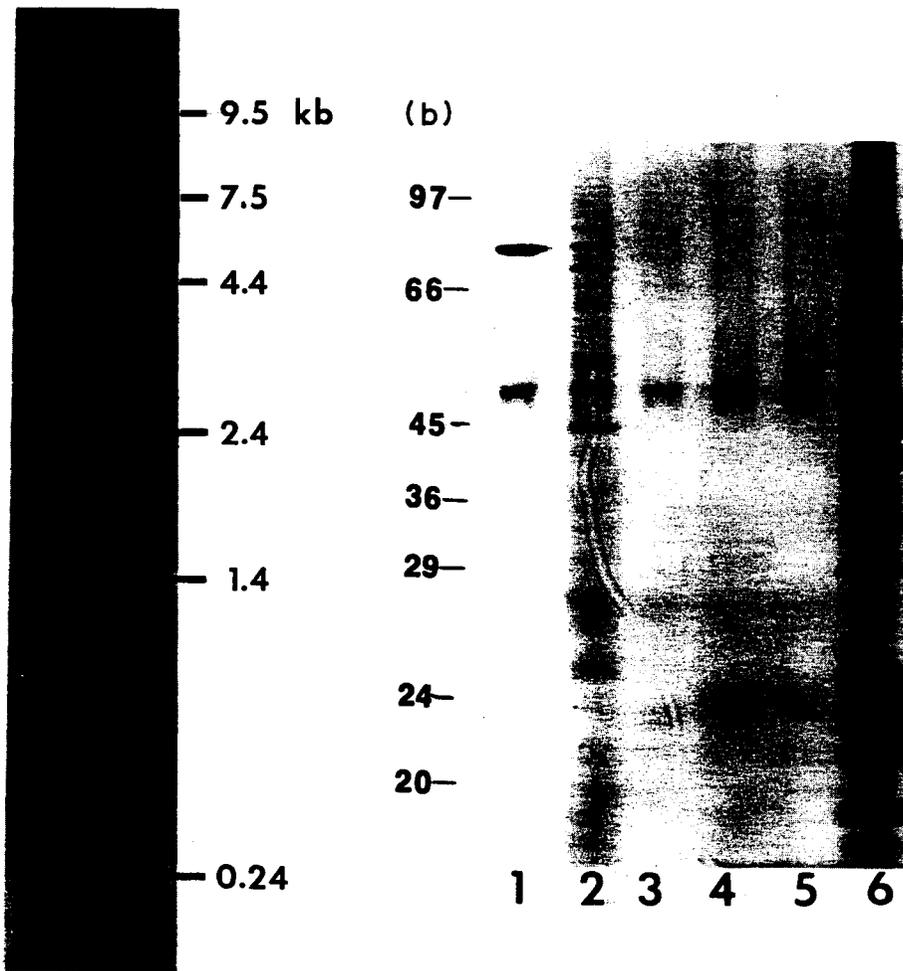


Fig. 1. (a) Autoradiograms of Northern blots of *M. sexta* day 6 fifth instar mRNA that was hybridized to ^{32}P -labeled insert DNAs from clones 119 and 201. RNA size markers are indicated in kilobases. (b) *In vitro* translation of mRNA hybrid-selected by clones 119 and 201. Single-stranded DNA corresponding to the two orientations of the insert DNA of clones 119 and 201 in M13 were immobilized on diazotized aminothiophenol paper and used to hybrid-select mRNA as described in Maniatis *et al.* (1982). The bound RNA was eluted and translated in a reticulocyte lysate using [^{35}S]methionine as the radioactive amino acid. The translation products were analyzed by electrophoresis in a 10% polyacrylamide gel followed by fluorography. The autoradiogram was exposed for 7 days. The RNAs used for translation were 1, RNA hybrid-selected by clone 201 minus strand; 2, total RNA; 3, no RNA; 4, RNA hybrid-selected by clone 201 plus strand; 5, RNA hybrid-selected by clone 119 plus strand; 6, RNA hybrid-selected by clone 119 minus strand. The migration positions and sizes (in kDa) of molecular weight markers are shown on the left. The presence of numerous bands in lane 6 with sizes < 79 kDa is due to overloading and premature termination of translation of RNA.



Figs 3 and 4. Captions on facing page.

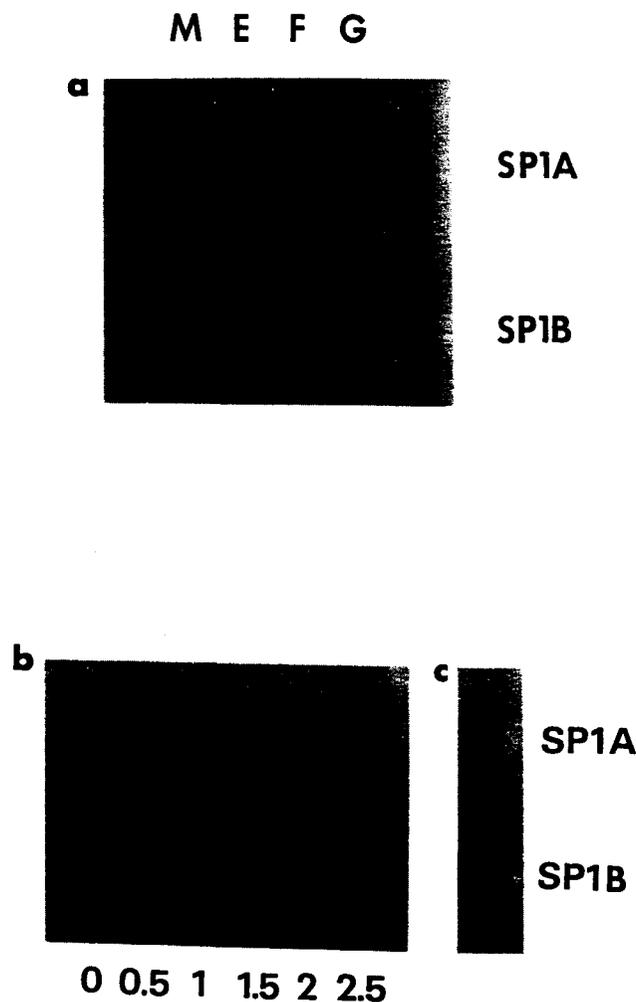


Fig. 5. Slot blot analyses of clone 119 (SP1A)- and clone 201 (SP1B)-specific mRNAs in 5 μ g total RNA from (a) muscle (M), epidermis (E), fat body (F), and midgut (G) from *M. sexta* on day 6 of fifth instar larvae, (b) day 0 through day 2.5 fat body of fourth instar larvae and (c) embryos.

Fig. 3. (See opposite page.) Cross-hybridization between insert DNA's from *M. sexta* clones 201, FSP, and 119. Plasmid DNAs from EcoR I-digested and Pst I-digested 201 and 119 clone DNAs were subjected to electrophoresis on agarose gels and blotted onto nitrocellulose membranes. Duplicate membranes were probed with 32 P-labeled insert DNA from the 400 bp Pst I fragment from clone 201 (panel A) and the insert DNA from clone 119 (panel B). The blots were hybridized as described in Materials and Methods and washed under conditions of moderate stringency using 2X SSC at 65°C. The autoradiogram was exposed for 3 h. Lanes: 1, FSP; 2, 201, and 3, 119.

Fig. 4. (See opposite page.) Southern blot analyses of *M. sexta* genomic DNA using insert DNA from clones 119 and 201 as probes. Aliquots (10 μ g) of DNA were digested with restriction enzymes Hind III (lane 1), EcoR V (lane 2), and EcoR I (lane 3). The sizes of marker fragments in kb are indicated on the right.

closely related to FSP and SP1A is a protein heretofore unidentified in *M. sexta*.

Clones 119 and 201 are single copy genes

The isolation of two cDNA clones with different DNA sequences from *M. sexta* that are related to the gene for the *B. mori* sex-specific storage protein SP1 indicates that at least two genes coding for storage proteins are expressed during development of the former species. A Southern blot analysis of *M. sexta* DNA probed with clones 119 and 201 was carried out to obtain an estimate of the gene copy numbers. As shown in Fig. 4, each probe detected only a single band in DNA digested with Hind III, EcoR V and EcoR I. Digestion with Pst I and Bam HI also resulted in single autoradiographic bands (data not shown). The most plausible interpretation of these data is that the genes corresponding to clones 119 and 201 exist as single copy genes in the *Manduca* genome. Probes 119 and 201 also detected DNA bands of different sizes in two of the restriction enzyme digests shown and failed to hybridize to bands detected by the other probe under the hybridization conditions used in this study. This result is consistent with the absence of cross-hybridization between insert DNAs from the two clones (Fig. 3).

Tissue-, stage- and sex-specific expression of M. sexta SP1A and SP1B genes

It was of interest to determine whether the two *M. sexta* genes corresponding to clones 119 (SP1A) and 201 (SP1B) exhibited similar tissue-, stage- and sex-specific expression. Total RNA was prepared from fat body, muscle, epidermal, and midgut tissues of day 6 fifth instar larvae. The amount of SP1A- and SP1B-specific mRNA in each tissue was quantitated by slot blot analysis using 5 microgram amounts of each RNA (Fig. 5). Fat body contained the highest

concentration of mRNA for both clones. Muscle, epidermis, and midgut contained minimal amounts of mRNA which could be attributed to a small degree of contamination with the fat body. It appears that the fat body is the major, if not the exclusive, site of synthesis of mRNA for both SP1A and SP1B.

To determine if mRNA from either clone was synthesized at developmental times prior to the fifth larval stadium, total RNA was isolated from embryos and from unsexed fourth instar larvae at 12 h intervals, applied to slot blots and probed with clones 119 and 201. Transcripts complementary to either clone were undetected in embryos or during the fourth larval stadium [Fig. 5(b) and (c)]. The results demonstrated that only the fifth instar fat body synthesizes mRNAs corresponding to clones 119 and 201.

Sex-specific expression of the two genes was investigated by isolating fat body RNA from male and female fifth instar larvae at one day intervals and carrying out slot blot analyses with clones 119 and 201 insert DNAs. As shown in Fig. 6, expression of both genes began around day 2 of the fifth larval stadium in the female, whereas in the male, transcripts were not detectable until later on day 4 at the time of wandering behavior. However, the patterns of accumulation of the mRNAs for the two genes were not parallel. In both males and females, mRNAs for SP1A persisted considerably beyond the time when SP1B-specific transcripts had already declined to very low levels.

Hormonal control of expression of the storage protein genes

The role of ecdysteroid in the expression of *M. sexta* storage protein genes was examined in unsexed fifth instar larvae that were ligated on day 2 between the first and second abdominal segments. In control abdomens, mRNA that hybridized with

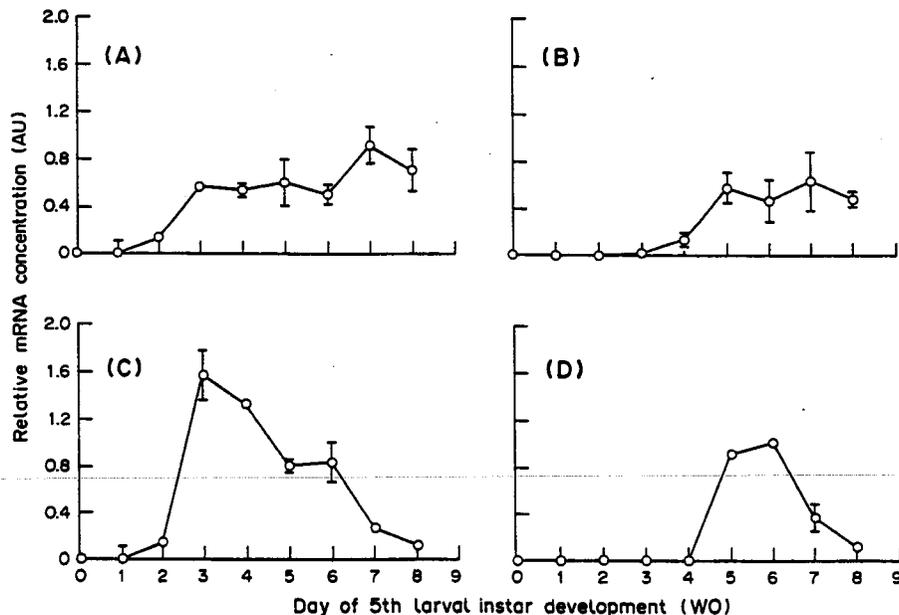


Fig. 6. Relative mRNA concentrations for SP1A (A), (B) and SP1B (C), (D) in fat bodies from female (A), (C) and male (B), (D) *M. sexta* fifth instar larvae as determined by slot blot hybridization. Six or more larvae were pooled for each mRNA preparation. $n = 3 \pm$ SEM. AU = absorbance units.

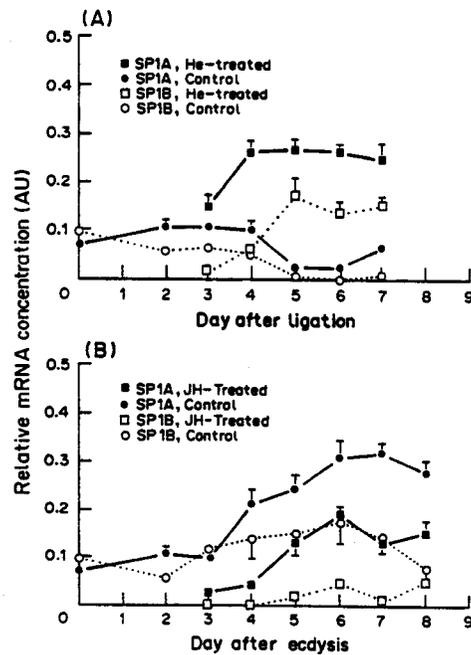


Fig. 7. (A) Effect of 20-hydroxyecdysone on RNA transcripts for SP1A and SP1B in *M. sexta* fifth instar isolated abdomens as determined by slot blot hybridization. Six or more larvae were pooled for each mRNA preparation. $n = 2 \pm 0.5$ range. AU = absorbance units. (B) Effect of fenoxycarb on RNA transcripts from SP1A and SP1B in *M. sexta* fifth instar larvae as determined by slot blot hybridization. $n = 2 \pm 0.5$ range. The arrows indicate the days of hormone treatment.

insert DNA from clones 119 and 201 remained relatively low from 3 to 8 days following ligation and no evidence of pupal cuticle formation was observed [Fig. 7(A)]. However, in 20-hydroxyecdysone-injected abdomens, the mRNA levels for SP1A increased, reached a maximum of approx. 11-fold higher than the mRNA levels of solvent-injected abdomens on the fifth day after ligation, and pupal cuticle formation occurred. Likewise, an increase in mRNA levels was observed for SP1B, which reached a level about 20-fold higher in hormone-injected abdomens. These data clearly demonstrated that ecdysteroid enhances by at least 10-fold mRNA expression of both SP1A and SP1B genes and that the temporal patterns of their hormonally stimulated expression in isolated abdomens are similar.

To determine if juvenile hormone also modulates the expression of the two *M. sexta* storage protein genes, a juvenile hormone mimic, fenoxycarb, was topically applied to unsexed fifth instar larvae at 2 and 3 days post ecdysis. Slot blot analyses of total fat body RNA from day 3 to day 8 revealed that substantial declines in transcripts for both SP1A and SP1B are caused by fenoxycarb treatment [Fig. 7(B)]. The results demonstrated that the JH mimic reduces the accumulation of SP1A and SP1B mRNAs.

DISCUSSION

During a search for cDNA clones representing proteins that are abundantly expressed during the

larval-pupal-adult transformation of *M. sexta*, two clones that are homologous with the gene for *B. mori* storage protein SP1 were identified. The DNA sequences of these two clones and the Southern blot analyses have demonstrated the presence of two distinctly different forms of methionine-rich storage proteins in *M. sexta*. These proteins have been named SP1A and SP1B to emphasize their similarity to the storage protein SP1 of *B. mori*. Like SP1, these two proteins are synthesized predominantly, if not exclusively, in the fat body.

SP1A transcripts persist at high levels late during the fifth larval stadium and their relative abundance is considerably greater than that of SP1B transcripts on days 7 and 8. This pattern of mRNA accumulation is also different from those of FSP and *B. mori* SP1 mRNAs. All of the SP1-type proteins apparently belong to a family of proteins that are synthesized by lepidopteran fat body late in larval development. They appear in the females earlier than in the males during the final larval stadium (or not at all as reported for *B. mori* males), are released into the hemolymph, and are subsequently taken up by the fat body prior to pupation. The storage proteins may ultimately be degraded and the amino acids utilized for metabolic reactions or pupal-adult protein synthesis during the pupal-adult transformation. The more rapid build-up of these proteins in the female relative to the male suggests a sexual dimorphic role in supplying the high nutritional needs of the female during metamorphosis and its reproductive cycle.

The protein product of *M. sexta* clone 201 (SP1B) is closely related to SP1 of *B. mori*, as revealed by the high percentage of identical residues (63%). The per cent identity between the aromatic amino acid-rich storage proteins, the arylphorins (SP2), from *M. sexta* (Willott *et al.*, 1989) and *B. mori* (Fujii *et al.*, 1989) is nearly the same, 62%. Therefore, the SP1B gene most likely corresponds to the gene for the *Bombyx* SP1 protein. Even though the protein product of clone 119, SP1A, shares only 37% identity with the amino acid sequence of *B. mori* SP1, we believe that the SP1A gene codes for a second SP1-like protein for the following reasons. First, the degree of similarity, as measured by the per cent identity of amino acid sequences between SP1A from *M. sexta* and SP1 from *B. mori* (37%), is slightly greater than the similarity between the SP1A protein and *M. sexta* arylphorins A or B (29 and 32%, respectively, Willott *et al.*, 1989). SP1A shares only 27% identity with the *B. mori* arylphorin. More importantly, the hormonal control and developmental pattern of SP1A expression closely parallel those of SP1B of *M. sexta* and SP1 of *B. mori* and not those of the arylphorins.

It is interesting to note that *M. sexta* SP1B protein is more closely related to *B. mori* SP1 protein than to *M. sexta* SP1A protein. The gene duplication that produced two copies of the gene for methionine-rich storage proteins must be more ancient than the time of separation of the genera *Manduca* and *Bombyx*. We would predict that, like *Manduca*, *Bombyx* and other insects must have two or more genes for the methionine-rich storage proteins. In fact, two multimeric proteins, one with a subunit apparent molecular mass of 85 kDa and the other of 89 kDa, were

detected in the hemolymph of *Hyalophora cecropia* (Tojo *et al.*, 1978). Two forms of SP1 with molecular masses of 620 and 730 kDa have also been detected in the hemolymph of *M. sexta* (Webb and Riddiford, 1988a, b). However, both of these aggregate forms showed only a single band of 79 kDa when analyzed by denaturing gel electrophoresis. We would like to suggest that the two forms may be composed of proteins corresponding to SP1A and SP1B proteins. However, in cultured fat body, only the 620 kDa protein could be detected, whereas in the medium, both forms were present. Webb and Riddiford (1988a) have suggested that the storage protein is initially assembled as a 620 kDa aggregate and is subsequently converted to the 730 kDa form during secretion. The nature of this conversion is unknown. It is conceivable that the two multimeric proteins assemble in different ways to produce aggregates of different sizes, even though their subunit molecular masses are the same.

In addition to their similarity to arylphorins, SP1A and SP1B proteins are also homologous to hemocyanins from a variety of arthropods, including horseshoe crab, spiny lobster, and tarantula spider (27–30% identity, data not shown, Kanost *et al.*, 1990). More than 80% of the residues have a positive contribution according to the McLachlan scoring index for related amino acids. The homology of the SP1 (methionine-rich)—and SP2 (arylphorin)—type storage proteins and an acidic juvenile hormone-suppressible protein to the hemocyanins may indicate that all of these arthropod genes have evolved from a common ancestor (Fujii *et al.*, 1989; Willott *et al.*, 1989; Jones *et al.*, 1990).

The mRNAs for both *M. sexta* storage proteins (SP1A and SP1B) accumulate in female fat body during the middle to late part of the fifth larval stadium feeding period. The longer persistence of SP1A mRNA late in the fifth larval stadium may be due to a greater stability of this mRNA relative to SP1B mRNA. Alternatively, synthesis of the SP1A mRNA may continue beyond the time when SP1B mRNA synthesis has shut down. Additional data are needed to determine how the non-coordinate accumulation of SP1A and SP1B genes is accomplished.

Riddiford and Hice (1985) found that mRNA of a female specific storage protein (FSP) of *M. sexta* appears in the female larval fat body at the time of JH decline prior to the onset of metamorphosis to the pupa, which is a pattern identical to that observed in this study for SP1A and SP1B. In allatectomized larvae, FSP mRNA appears precociously, whereas its appearance is prevented by application of the JH mimic, methoprene, indicating that JH down-regulates the appearance of FSP mRNA (Webb and Riddiford, 1988a, b). In contrast, when the supply of both JH and ecdysteroid was reduced by abdominal ligation, fat body from fifth instar female larvae failed to show an early appearance of FSP mRNA, indicating that the removal of JH alone is not sufficient to bring about expression of FSP mRNA. In the present study injection of ecdysteroid into ligated abdomens resulted in the elevation of both SP1A and SP1B mRNAs, whereas administration of a juvenoid to mature larvae suppressed their levels. These results

are consistent with the hypothesis that the two *M. sexta* genes exhibit positive regulation by ecdysteroid and negative regulation by JH. However, the hormonal effects on the accumulation of transcripts for the two genes are not identical and are manifested in a sex- and tissue-specific fashion. In addition to its transcriptional effects, ecdysteroid also promotes uptake of the methionine-rich proteins by the fat body in the latter part of the last larval stadium (Tojo *et al.*, 1981; Webb and Riddiford, 1988a, b). Further study of these proteins may lead to a better understanding of their physiological functions, as well as the identification of other factors that are fundamental to the regulation of storage protein gene expression during insect development.

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