

Identification of a Novel Type of Silk Protein and Regulation of Its Expression*

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The silk of lepidopteran insects has been studied extensively as proteins of two categories: the fibroins, which are produced in the posterior section of silk glands, and the sericins, which are secreted in the middle section. We now describe a third category that is named seroins to accentuate the fact that both the sericin- and the fibroin-producing cells participate in seroin secretion. Using a probe derived from the N-terminal sequences of a 23-kDa components of *Galleria mellonella* silk, we isolated silk gland-specific cDNA encoding 167 amino acids, of which 17 constitute the signal peptide. The following 14 residues match the N-terminal sequences of the 23- and 22.5-kDa silk protein. The reaction of these proteins with concanavalin A and the presence of two glycosylation sites in the seroin peptide sequence indicate that seroin is secreted in two forms that both contain a mannose-rich sugar moiety. Seroin is distinguished from other silk proteins by high proline content (34 residues or 20.26% by weight), lack of cysteines, and the presence of two kinds of short amino acid repeats. The seroin gene is expressed in both the posterior and middle silk gland sections. The expression fluctuates during development in correlation with the feeding regime and the changes in hormone titers: seroin mRNA is high in the silk glands of feeding larvae, declines at ecdysis, reaches a maximum during cocoon spinning, and thereafter rapidly drops to an undetectable level. *In vivo* and *in vitro* experiments showed that the drop is caused by ecdysteroid hormones and is prevented by juvenile hormones. N-terminal sequencing of several silk proteins of *Bombyx mori* revealed that the 8- and 13-kDa proteins share 5 or 6 out of 10 identified amino acids with the N terminus of *Galleria* seroin and obviously represent seroin homologues. The result suggests that seroin-type proteins are a general component of lepidopteran silk.

The labial glands of insects usually produce saliva used in digestion, but in the larvae of many caddisflies (Trichoptera), moths, and butterflies (Lepidoptera) they secrete a solid fiber,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF009828.

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the silk, and are therefore called the silk glands. The posterior sections of the two silk glands (PSG)¹ generate heavy chain fibroin (H-fibroin), light chain fibroin (L-fibroin), and protein p25, which are assembled into an insoluble fibroin thread. The middle silk gland sections (MSG) secrete several sericins that provide this thread with sticky coating. The anterior silk gland sections merge into an outlet terminating on the spinneret from which a single silk cord is spun (1).

The analysis of silk proteins is difficult because only some of the sericins are water-soluble (see Fig. 2). Most of our knowledge of silk comes from molecular biology studies on the major commercial silk producer, the domestic silkworm, *Bombyx mori*. It has been demonstrated that H-fibroin, a 350-kDa polypeptide (2), is linked by disulfide bonds to the 25-kDa L-fibroin and is also associated with two p25 proteins of 27 and 30 kDa (3). The sericins from MSG include several glycoproteins with apparent molecular masses ranging from 65 to 400 kDa (4, 5). Sericins are characterized by unbalanced amino acid composition, with serine accounting for 16–42% of all amino acids (6). It has been proven that genes for H-fibroin (7), L-fibroin (8), and p25 protein (9) are specifically expressed in PSG, whereas the two sericin genes, *Ser1* (10) and *Ser2* (11), are active in MSG.

Homologous silk components were discovered at the gene or protein level in another lepidopteran, the waxmoth *Galleria mellonella*. The L-fibroin and p25 of this species were identified by partial sequencing of isolated silk proteins and as cDNA clones; the deduced amino acid sequences proved to be 43 and 51% identical with *Bombyx* L-fibroin and p25, respectively, with conserved positions of all cysteine residues (three in L-fibroin, eight in p25) (12, 13). A lower amino acid conservation, but a similar tandem distribution of the amino acid repeats, and virtually identical gene organization were found in the *H-fibroin* gene.² Two sericin genes, resembling *Ser1* and *Ser2* of *Bombyx*, were identified in *Galleria* as MSG-specific cDNAs, which were partially sequenced (14).

In addition to the major silk proteins described above, several components of low molecular weight were detected in the silk of both *Bombyx* and *Galleria* (15, 16). Some of them occur in the silk in considerable amounts (17), suggesting that they may have important functions, possibly similar to those of L-fibroin and p25. The L-fibroin and p25 proteins are essential for the transformation of H-fibroin from a liquid column to the solid thread during spinning (18, 19).

The possibility cannot be excluded that some of the unknown

¹ The abbreviations used are: PSG, posterior section(s) of silk glands; MSG, middle section(s) of silk glands; H-fibroin, heavy chain fibroin; L-fibroin, light chain fibroin; PAGE, polyacrylamide gel electrophoresis; nt, nucleotide(s); 20E, 20-hydroxyecdysone; JH, juvenile hormone(s); tsp, transcription start point; UTR, untranslated region.

² M. Žurovec, C. Yang, D. Kodrík, and F. Sehnal, unpublished observations.

silk components are smaller forms of the larger silk proteins. For example, *Galleria* gene *p25*, which yields the glycosylated silk proteins of 29 and 30 kDa (13), encodes a secretory peptide of only 23 kDa that could occur in the silk. We decided to investigate whether silk proteins of corresponding sizes did not include the unglycosylated version of *p25*. In course of this work, we identified a novel silk protein type that differs from all the others structurally and by the site of production. It is called seroin to illustrate that it is an ingredient of both the sericin and the fibroin silk fractions. In this paper, we report the cDNA sequence that encodes *Galleria* seroin and show that this peptide is secreted as two water-insoluble glycoproteins of 22.5 and 23 kDa. The spatial, developmental, and hormonal control of *seroin* gene expression is described. We also provide evidence for the presence of proteins with seroin N-terminal sequence in the silk of *Bombyx*.

EXPERIMENTAL PROCEDURES

Insects and Tissue Handling—Larvae of the waxmoth, *G. mellonella* L., were reared on a semiartificial diet (20) at 30 °C, and those of the domestic silkworm, *B. mori* (Japanese hybrid NO2 × CO2) were reared on mulberry leaves at room temperature. Silk glands were dissected from water-anesthetized larvae, and in most cases they were immediately frozen in liquid nitrogen. In some cases, the middle and posterior parts of silk glands were separated, and muscles, fat body, and the remaining body carcass were also preserved at -80 °C. Some insects were allowed to spin cocoons that were used for protein analysis.

Polypeptide Separation and Amino Acid Sequencing—Clean cocoons were chopped to small pieces, and samples of 200 mg were either boiled four times for 1 min in 2 ml of water (each washing was followed by a short centrifugation) or just rinsed in cold water. Silk proteins were then solubilized by soaking the samples for 48 h in 1.5 ml solutions containing 10 mM Tris (pH 7.0), 2% SDS, 8 M urea, and 5% 2-mercaptoethanol. Insoluble heavy chain fibroin was removed by centrifugation (5 min at 60 × g) and 100- μ l aliquots of the supernatant were analyzed by SDS-PAGE on 20% gel. The proteins were visualized with Coomassie Blue staining and electroblotted onto a polyvinylidene difluoride membrane, from which selected bands were cut out and sent for N-terminal microsequencing (21) to the commercial facility of the Medical College of Wisconsin (Milwaukee, WI). Proteins electroblotted onto a nitrocellulose membrane were probed for the presence of sugars with the lectins concanavalin A, lentil agglutinin, peanut agglutinin, and wheat germ agglutinin (all from Lectinola, Prague), which were conjugated to horseradish peroxidase (22). Conjugates bound to the blots were revealed with 3,3-diaminobenzidine.

Isolation of cDNA Clones and Nucleotide Sequencing—*Galleria* silk gland cDNA library in λ gt10 (14) was screened with degenerate 30-mer oligoprobe that was derived from the N-terminal sequences of the 23-kDa silk proteins. The probe (10 pmol) was end-labeled with 50 μ Ci of [γ -³²P]ATP; free nucleotides were removed by chromatography on Sephadex G-25. Hybridization was performed at 36 °C in 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, and washing was at room temperature twice in 2 × SSPE, 0.1% SDS and twice in 1 × SSPE, 0.1% SDS. Positive cDNA clones were purified, and the inserts were subcloned in pBlue-script SK (+/-) (Stratagene) and sequenced with the Sequenase version 2.0 sequencing kit (Amersham Pharmacia Biotech). The library was rescreened with radiolabeled (Random Primed Labeling kit, Amersham Pharmacia Biotech) 5'-fragment (356 nt) of a cDNA insert to isolate the full-length cDNA. The same fragment was employed as a probe in all subsequent nucleic acid analyses. DNASTAR software and FASTA, BLAST, and other network services were used to analyze the sequencing data.

Primer Extension—Primer extension was carried out in the standard way³ using the 19-mer oligonucleotide shown in Fig. 1. The annealing reaction with 50 μ g of total RNA from silk glands of day 3 last instar larvae was done at 32 °C for 16 h.

RNA and Genomic DNA Analysis—Northern and dot blot analysis were performed as described previously (24), using the 356-nt fragment as probe. Relative amounts of hybridized RNA were assessed by scanning the autoradiographs with a CAMAG TLC Scanner II densitometer. The maximal content, which was found in mobile prepupae, was taken

as 100%, and values established at other times were expressed relative to this amount. Effects of hormones and protein synthesis inhibitors on the mRNA content were quantified by measuring radioactivity of post-hybridized Northern blots of the corresponding RNA bands. Radioactivity in other areas was regarded as background and was subtracted. RNAs from the control and experimental glands were electrophoresed in parallel lanes and transferred onto one blot. The maximal value established on each such blot was taken as 100%, and other values were expressed as its percentage. Each analysis was repeated three or four times, and the results were pooled.

Aliquots of the genomic DNA from day 3 last instar larvae were digested with *EcoRI*, *EcoRV*, *SalI*, and *XbaI*, respectively, and subjected to standard Southern analysis with *seroin* cDNA probe.

Hormone Applications—*Galleria* prepupae at an advanced stage of cocoon spinning 48–60 h before pupal ecdysis were topically treated with juvenile hormone bioanalogue W-328 (25). Each insect received 1 μ g of W-328 in 2 μ l of acetone, and the controls were treated with pure solvent. Silk glands were usually dissected 12 h before pupal ecdysis.

Most hormonal treatments were done *in vitro*. Silk glands were dissected from day 6 last instar larvae (onset of cocoon spinning) and transferred to amino acid-enriched Grace's medium (Life Technologies, Inc.), which was supplemented with streptomycin (20 μ g/ml) and either kanamycin sulfate (0.05 mg/ml) or ampicillin (10 units/ml). A pair of silk glands was placed in 0.5 ml of medium in a glass vial coated with polyethylene glycol 20,000 (26). Hormones 20-hydroxyecdysone (20E) (5 μ g/ml) and/or juvenile hormone II (20 ng/ml) were added to the medium in acetone (5 μ l of acetone per 0.5 ml of medium). The culture was carried out under gentle shaking for 24 h at 27 °C; the medium was replaced at 12 h. In a few cases, cycloheximide (40 μ g/ml) or anisomycin (10 μ g/ml), which efficiently inhibited protein synthesis in explanted silk glands (27), were dissolved in the medium just before use.

RESULTS

Characterization of *Galleria Seroin*—N-terminal sequencing of a 23-kDa protein from the solubilized cocoons revealed a 14-amino acid sequence AFV(R?)VDDDDNNSFPK. The identity of the fourth residue with Arg was doubtful, but the remaining sequence clearly showed that we were dealing with an unknown silk protein. An oligoprobe (A/G)TT (A/G)TT (A/G)TC (A/G)TC (A/G)TC IAC NC(G/T) IAC (G/T)AA NCG was designed on the basis of the first 10 amino acids and used to screen about 2 × 10⁴ plaques of the silk gland-specific cDNA library prepared previously (14). All three cDNA clones, which were isolated in the first screen, contained an open reading frame that included appropriate amino acid sequence. A cDNA insert of 356 base pairs was used to rescreen the library, and a nearly full-length cDNA clone was identified among 1.5 × 10⁵ plaques. The nucleotide sequence of this cDNA contained several ATG codons; however, only the translation product initiated at the first ATG included the desired amino acid sequence. The first ATG codon was also in proper position for translation initiation (an A at the critical -3-position and a G at the +4-position) (28), and the 5'-flanking region was similar to the translational start site consensus identified in *Drosophila* (29).

The open reading frame initiated by the first in frame ATG consisted of 501 nt and ended with a TAA termination codon in nucleotide positions 564–566 (Fig. 1). A putative translation product of 167 amino acid residues apparently included a signal peptide of 17 residues, because amino acid sequence corresponding to the N terminus of the secreted 23-kDa silk protein began at residue 18. The amino acid arrangement around residues 17 and 18 complied with rules for signal peptide cleavage (30).

The sequence established by N-terminal sequencing of the 23-kDa silk protein differed from that of the deduced peptide in 2 of 14 amino acids. Since all analyzed cDNA clones encoded identical N terminus, the mismatches were obviously due to errors in peptide sequencing. To make sure that the identified cDNA corresponded to the 23-kDa protein, we repeated sequencing of 10 N-terminal amino acid residues. N-terminal sequences were also established for the adjacent 22.5-kDa protein and for other silk proteins discerned in the region between

³ C. Yang, X. Teng, M. Žurovec, K. Scheller, and F. Sehnal, submitted for publication.

TABLE I

Sequence of 11 N-terminal amino acids of low molecular silk proteins extracted from the cocoons of *G. mellonella* and *B. mori*

Silk protein	Genus	N-terminal protein sequence	Overlap %
L-fibroin	<i>Galleria</i>	G P A N N V V R P P R	
L-fibroin	<i>Bombyx</i>	G P P S P I Y R P C Y	36
p25	<i>Galleria</i>	S V V I S Q D N I N N	
p25	<i>Bombyx</i>	S V T I N Q Y S N N E	45
Seroin (18 kDa)	<i>Galleria</i>	G F V W V D D D N N S	
Seroin (13 kDa)	<i>Bombyx</i>	G F V ? E D D D D L F	60
Seroin (8 kDa)	<i>Bombyx</i>	G F V ? E D D N F P G	50
16 kDa protein	<i>Galleria</i>	G K G C D D G G D G K	

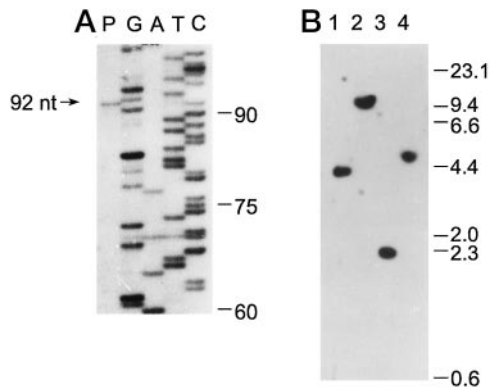


FIG. 3. Preliminary characterization of the *seroin* gene. A, primer extension mapping of the transcription start of the *seroin* gene. A 5'-radiolabeled 19-mer oligonucleotide (cf. Fig. 1) and 50 μ g of silk gland RNA were used to synthesize, with the aid of Moloney murine leukemia virus reverse transcriptase, the complementary DNA strand of 92 base pairs (lane P, arrow). The G, A, T, and C sequencing ladders are included; the length of analyzed sequence is shown on the right. B, Southern blot analysis of 5 μ g of *Galleria* genomic DNA that was digested with *Xba*I (lane 1), *Sal*I (lane 2), *Eco*RV (lane 3), and *Eco*RI (lane 4), respectively, and hybridized to the radiolabeled 356-nt fragment of *seroin* cDNA. Size markers are indicated on the right.

was expressed abundantly in the MSG and, to a lesser extent, in the PSG, but not in the other tissues (Fig. 4). Also, no signal was detected in the Northern blots of total RNA prepared from larval carcasses deprived of the silk glands but containing all other organs (data not shown). These results invite the conclusion that expression of the *seroin* gene is confined to the silk-secreting regions of silk glands.

The ratio of *seroin* mRNA in MSG versus PSG was similar in five randomly selected stages of the penultimate and last larval instars. Developmental changes in the relative content of *seroin* mRNA in the whole silk glands were examined in the course of the penultimate and last larval instars. We found that *seroin* mRNA accumulated in the feeding penultimate instar larvae and declined at molt to the last instar larvae (Fig. 5). A rapid postecdysial increase of *seroin* mRNA content coincided with the resumption of feeding. Within 24 h of the last instar, the relative amount of *seroin* mRNA reached a higher level than was the penultimate instar maximum. The onset of cocoon spinning at 120 h of the last instar was associated with another rise of *seroin* mRNA to an absolute peak in mobile prepupae. Following cocoon completion, the silk glands turned fragile, and their milky appearance signified apoptosis. The content of *seroin* mRNA rapidly dropped to a barely detectable level.

Insect development is known to be regulated by ecdysteroids and juvenile hormones (JH). Possible roles of these hormones in the control of *seroin* expression were examined by subjecting silk glands to aberrant hormonal situations. Silk glands developed in virtual absence of JH for most of the last larval instar

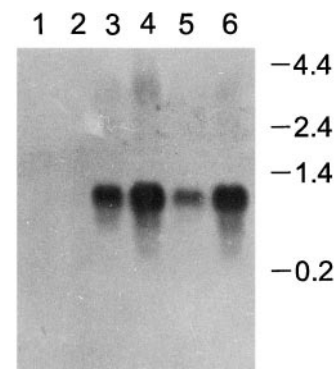


FIG. 4. Tissue-specific expression of the *seroin* gene. Northern blots of 10 μ g of total RNA isolated from muscles (lane 1), fat body (lane 2), middle silk gland (lanes 4 and 6), and posterior silk glands (lane 5) or 5 μ g of total RNA from middle silk gland (lane 3) were hybridized to the radiolabeled 356-nt fragment of *seroin* cDNA. Positions of an RNA ladder are given on the right.

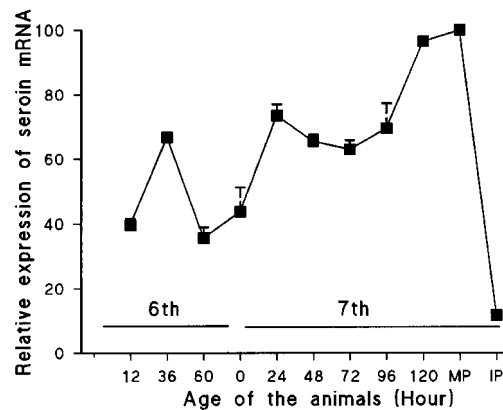


FIG. 5. Developmental profile of the relative content of *seroin* mRNA. Samples of 2, 0.5, and 0.125 μ g of silk gland RNA were dot-blotted on Hybond-N membrane and hybridized with radiolabeled *seroin* cDNA probe. The amounts of hybridized label were assessed by scanning the fluorographs and averaging the values of four or five independent assays. Values established at indicated times of the penultimate (sixth) and last (seventh) larval instars are expressed relative to the maximum mRNA content in mobile prepupae (equal to 100%). The ages of animals are given in hours after ecdysis into the respective instar. MP, mobile prepupae; IP, immobile prepupae (144 and 168 h after the last ecdysis, respectively).

(32), and in our experiment we took advantage of this fact by challenging them with the JH analog W-328, which was administered to mobile prepupae. The untreated insects reached the stage of immobile prepupae within 24 h (point IP in the graph in Fig. 5), and the *seroin* mRNA became undetectable. The control insects, which were treated with acetone, appeared as immobile prepupae 36 h after the treatment, their silk glands showing signs of apoptosis and no detectable levels of *seroin* mRNA (Fig. 6, lane 4). By contrast, the silk glands of insects treated with JH analog retained their integrity for at least 70 h when the insects were sacrificed. Some of the treated insects were at the stage of immobile prepupa, whereas others had already ecdysed to pupae with slightly shortened wings and appendages, a typical JH effect. The silk glands of all of these animals contained considerable amounts of *seroin* mRNA (Fig. 6, lanes 1–3). The JH analog prevented silk gland degradation, which normally ensues after the pupal ecdysis and thus averted the termination of *seroin* gene transcription.

Silk glands explanted from day 6 last instar larvae, the time when the content of *seroin* mRNA is rising (see Fig. 5) and the titer of ecdysteroids is low (33), were used to test the effects of hormones *in vitro*. The addition of 20E to the culture medium, at a dose mimicking ecdysteroid concentration in the hemo-

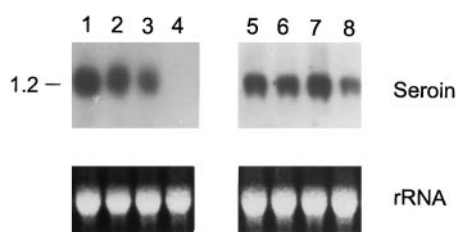


FIG. 6. Effects of juvenile and ecdysteroid hormones on seroin expression. Juvenoid W-328 was applied on mobile prepupae, and the silk glands were dissected 70 h later when the insects were approaching ecdysis (lanes 1 and 2) or ecdysed to slightly deformed pupae (lane 3); silk glands of controls were analyzed at a comparable physiological stage 36 h after the acetone treatment (lane 4). Silk glands explanted from day 6 last instar larvae were cultured for 24 h *in vitro* in the presence of ethanol, which was used as hormone solvent (lanes 5 and 7), 20-hydroxyecdysone (5 μ g/ml) (lane 8), and 20-hydroxyecdysone plus juvenile hormone II (20 ng/ml) (lane 6), respectively. In all cases, 5 μ g of total RNA from the silk glands were hybridized to the seroin cDNA probe, which revealed the 1.2-kilobase pair band of seroin mRNA. The rRNA fractions stained with ethidium bromide are shown to document equal sample loading.

lymph of the pupating insects, caused a decrease of seroin mRNA (Fig. 6, lane 8 versus lanes 5 and 7). The decline was alleviated when the medium containing 20E was also supplied with juvenile hormone II (Fig. 6, lane 6).

When the level of seroin mRNA in the total RNA of silk glands cultured in the presence of the solvent was taken as $100 \pm 17\%$, the level measured in the 20E-treated medium amounted to $42 \pm 11\%$. However, in the presence of cycloheximide and anisomycin, the content of the mRNA was reduced by 20E only to 70 ± 14 and $70 \pm 9\%$ of the control value, respectively.

Identification of Seroin Homologues in Bombyx—Since we suspected that the silk of *B. mori* might contain a seroin homolog, the N-terminal sequences of several small size components of the *Bombyx* cocoon silk were established in a similar way as in *Galleria* silk proteins. Eleven N-terminal amino acids identified in the 8- and 13-kDa *Bombyx* silk proteins were nearly identical to the N terminus of *Galleria* seroin (Table I). Interestingly, the residue in position 4 could not be read, similarly as with *Galleria* seroin. Despite this, the homology between *Galleria* seroin and the *Bombyx* 8- and 13-kDa proteins is higher than between the L-fibroin and p25 counterparts in the two species (Table I).

DISCUSSION

Seroin Proteins—Structural characterization of individual silk components is a necessary prerequisite for elucidating their roles in the production, physical properties, chemical resistance, and biological functions of silk. In this paper, we describe the existence of a distinct class of small silk components, the seroins, that differ from the bulk of large size silk proteins structurally and in that their production occurs both in PSG, where fibroins are produced, and in MSG, where fibroin coating by sericins is provided. This dual origin is emphasized by the term “seroin.”

The secreted (after the signal peptide removal) seroin peptide of *Galleria* (Fig. 1) differs from all known silk proteins by its high proline content (34 residues or 20.26% by weight) and lack of cysteine. More than a half of the proline residues are contained in two copies of a PPLPQPPPL repeat and in three copies of a PPI motif. Acidic residues prevail over the basic ones, and the calculated pI of seroin therefore equals 4.93. The N-terminal amino acid sequence of the silk proteins reveals that the seroin peptide is present in silk as 22.5- and 23-kDa glycoproteins. Their reaction with concanavalin A (Fig. 2, lanes 2 and 3) indicates the presence of mannose-rich carbohydrates.

The nonglycosylated form of seroin, which would migrate as a 16-kDa protein, is not secreted. This situation is reminiscent of the p25 gene product that also occurs in the silk as two mannose-rich glycoproteins whose apparent size is, by 6–7 kDa, larger than the molecular mass of the deduced peptide.

The relatively high content of polar amino acids and the glycosylation should make seroins water-soluble. However, washing silk with hot water did not reduce the content of the 22.5- and 23-kDa proteins (Fig. 2, lanes 1 and 2). This observation suggests that seroins are liberated from the silk only when most of the silk components are dissolved.

The function of seroin is elusive. Since the high proline content is reminiscent of the proline-rich antimicrobial peptides (23), we suspect that seroin may be responsible for silk resistance to molds and bacteria.

Seroin Gene and Its Expression—Our data indicate that there is one copy of the seroin gene present in the genome, similarly to other lepidopteran silk genes. As in other silk genes, seroin expression exhibits a high degree of spatial and temporal specificity. However, the simultaneous expression of seroin in PSG and MSG is unique.

Down-regulation of silk genes by ecdysteroids was previously illustrated in experiments with isolated abdomens of postfeeding *Galleria* larvae. The removal of the head and thorax deprived the insects of their source of ecdysteroids and most other hormones, but the abdomens survived and the silk glands remained functional for many weeks. The contents of sericin mRNAs (24) and p25 mRNA³ declined gradually in such preparations, but application of a molt-inducing 20E dose elicited their rapid disappearance. These experiments could not reveal, however, if 20E acted directly on the silk glands or if the effect was mediated by some other tissues, for example the fat body that provides silk glands with nutrients. In the present study, we demonstrate that the content of seroin mRNA declines when explanted silk glands are exposed to 20E in the absence of any other tissue (Fig. 6). As expected, the decline is much lower in the presence of protein synthesis inhibitors, indicating that the seroin gene is not the primary target of the hormone.

The titer of ecdysteroids rises and the activity of silk glands is diminished during each ecdysis (1, 7). In a previous study, we stated that considerable amounts of sericin mRNAs persist in the silk glands during larval ecdyses but become undetectable during the pupal ecdysis, when the silk glands initiate apoptosis (24). Administration of a JH analog to larvae before pupal ecdysis prevents apoptosis, and the sericin mRNA remains present. We now show that JH exerts such a protective effect on the seroin mRNA in explanted silk glands in the absence of any other organ (Fig. 6).

The results of our *in vitro* tests demonstrate that both ecdysteroids and JH act directly on the silk glands. An enhanced ecdysteroid concentration suppresses expression of the seroin and other silk genes, and in the absence of JH it elicits silk gland degeneration; the latter effect is prevented and silk gene expression is restored when exogenous JH or a JH analog is added.

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