**Organization of Serpin Gene-1 from Manduca sexta**

**EVOLUTION OF A FAMILY OF ALTERNATE EXONS ENCODING THE REACTIVE SITE LOOP*§

(Received for publication, May 28, 1996, and in revised form, August 30, 1996)

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*Manduca sexta* serpin gene-1 encodes a family of serpins whose amino acid sequences are identical in their amino-terminal 336 residues but variable in their carboxyl-terminal 39–46 residues, which includes the reactive site loop (Jiang, H., Wang, Y., and Kanost, M. R. (1994) *J. Biol. Chem.* 269, 55–58). Here, we report the gene’s complete nucleotide sequence and exon-intron structure. A unique characteristic of this gene is its exon 9, which is present in 12 alternate forms between exons 8 and 10. Isolation and characterization of cDNA clones containing exons 9C, 9H, and 9I, which were not found previously, indicate that all 12 alternate forms of exon 9 can be utilized to generate 12 different serpins. The splicing pathway apparently allows inclusion of only one exon 9 per molecule of mature serpin-1 mRNA. Analysis of exon-intron border sequences reveals unique features that may be involved in regulation of RNA splicing. The exon 9 region has apparently evolved through rounds of exon duplication and sequence divergence. The exons near the center of the region may have evolved recently, whereas the outermost exons are the most ancient. Exons 9G and 9H were duplicated as a pair from exons 9E and 9F, an event that may have occurred more than once in the history of this gene.

The serpin superfamily contains a large number of proteins that function as inhibitors of serine proteinases as well as proteins related in sequence which are not inhibitors (1). Serpins are typically 370–390 amino acid residues long, with a reactive site loop 30–40 residues from the carboxyl terminus. This loop, exposed at the surface of the protein, is the site of interaction between serpins and the serine proteinases they inhibit. The serpin reactive site loop binds to the active site of a target proteinase in a manner similar to the binding of a substrate, forming a very stable serpin-proteinase complex. Formation of this complex involves a specific peptide bond in the reactive site loop, the scissile bond (designated \( P_1'-P_1 \)). The amino acid sequence of the reactive site loop determines an inhibitor’s selectivity. Altering the reactive site loop sequence, particularly at the \( P_1 \) position, can cause dramatic changes in the proteinase selectivity of a serpin (1). Comparisons of serpin sequences have demonstrated that the reactive site loop and adjacent sequence is the least conserved region of the proteins. It has been suggested that after duplications of serpin genes, rapid evolutionary change of the reactive site loop region provides new inhibitor selectivities that may have value during natural selection (2, 3).

At least nine different serpins are present in mammalian plasma. They regulate the activity of serine proteinases involved in diverse physiological functions such as blood coagulation, fibrinolysis, complement activation, and inflammatory responses (1). Serpins have also been found in the hemolymph of invertebrates, including three groups of arthropods: insects, crayfish, and horseshoe crabs (4). These arthropod serpins have 12–30% amino acid sequence identity with various mammalian serpins. Like their mammalian counterparts, invertebrate serpins may regulate proteinases released from blood cells during inflammation-like processes, and they may regulate pathways of proteinase cascades that ultimately activate enzymes involved in blood coagulation and melanization (4–6).

Study of serpins from two species of lepidopteran insects, *Manduca sexta* (tobacco hornworm) and *Bombyx mori* (silk-worm), has revealed a novel genetic mechanism for generating diversity of serpin reactive site loops. Amino acid sequences of two serpins isolated from *B. mori* are identical in their first 336 amino acid residues but differ in sequence from residue 337 to the carboxyl terminus (7). We isolated a large number of serpin cDNA clones from *M. sexta* and found the same phenomenon (8). In 38 cDNA clones, the DNA sequences of the 5’-untranslated region, the sequence encoding a secretion signal peptide, and the first 336 amino acid residues of the mature protein were identical. Beyond this point the sequences differed from clone to clone, with 10 distinct sequence variants encoding the carboxyl-terminal 39–46 residues, which includes the reactive site loop. The 3’-untranslated sequences following the translation stop codon were again identical in all of these serpin cDNAs.

The occurrence in *M. sexta* of serpin cDNAs with a variable region encoding the reactive site loop between two constant regions appears to be due to mutually exclusive alternate exon use (8). The variable sequence is encoded by multiple versions of the ninth exon of *M. sexta* serpin gene-1. We present here the complete nucleotide sequence of *M. sexta* serpin gene-1, in which we have identified 12 alternate versions of exon 9. We have also isolated cDNAs corresponding to all of these exon 9 variants, indicating that they are all expressed. An analysis of the region of the gene containing the exon 9 variants suggests
a pathway for the duplication and diversification of the alternate exons during evolution of the gene.

**Experimental Procedures**

**Isolation of Genomic Clones—**A *M. sexta* genomic DNA library in λ phage EMBL3 (9) was screened by hybridization with a α32P-labeled *M. sexta* serpin-1B (previously called alaserpin) cDNA clone (10) by methods described by Sambrook et al. (11). A total of 105 recombinant plaques (three genomic equivalents) from the library was screened. Positive plaques were purified, and DNA was prepared from plate lysates (12). λ phage DNA from clone λE1 was digested with SallI, and the resulting fragments were subcloned into plasmid pTZ18U. A 2.7-kb *SalI-EcoR I* fragment from clone λA2 was subcloned into plasmid pBluescript(KS).

A polymerase chain reaction (PCR) was used to obtain a 5-kb DNA fragment of the serpin gene-1 which was not represented in the λ clones. Oligonucleotide primers were designed, based on sequence data obtained from regions near the ends of clone λE1 (primer E: 5' -CT- TAC TGG GAT CCA TAG AC-3') and clone λA2 (primer A: 5'-CGA T- TGAG TCT AGA AGG A-3'). The PCR was carried out using a 1:1 mixture of *Taq* and *Pfu* DNA polymerases (Stratagene). Thermal cycling conditions were: 95 °C, 20 s; 53 °C, 60 s; 72 °C, 4.5 min for 30 cycles. The resulting DNA product was isolated by agarose gel electrophoresis and cloned into pGem-T vector (Promega).

**Southern Blot Analysis of Genomic DNA—**Genomic DNA was isolated from a single *M. sexta* larva (13). Samples of the DNA (10 µg) were digested with restriction enzymes at 37 °C for 6 h. The resulting fragments were separated by electrophoresis in a 1% agarose gel, trans- digested with restriction enzymes at 37°C for 6 h. The resulting fragments were separated by electrophoresis and cloned into pGem-T vector (Promega).

**Sequence Analysis—**Serpin cDNA clones were sequenced using subcloned restriction enzyme fragments, subclones (U.S. Biochemical Corp.). cDNA inserts were sequenced using T7 or T3 dideoxynucleotide method (14) using modified T7 DNA polymerase (Stratagene). Thermal cycling conditions were: 95 °C, 20 s; 53 °C, 60 s; 72 °C, 4.5 min for 30 cycles.

**Computer-assisted Analysis of Sequence Data—**Sequence analysis was performed using the GCG Sequence Analysis Software Package version 7.3.1 (16) and IBI Pustell programs.

**results**

**Nucleotide Sequence of *M. sexta* Serpin Gene-1—**We screened a *M. sexta* genomic DNA library with a serpin-1B cDNA probe (previously called alaserpin) (10). We isolated three overlapping λ clones (λC2, λA1, λE1) and another clone (λA2) that did not overlap with this group (Fig. 1). Restriction mapping and Southern blot analysis demonstrated that the 5' end of the cDNA hybridized with clone λE1 and that clone λA2 hybridized with the 3' end of the cDNA (data not shown). We used sequence from the ends of λE1 and λA2 to design primers for a PCR to obtain the missing DNA fragment linking these two clones. Primer E was from a sequence 187 nucleotides from the 3' end of λE1; primer A was from a sequence 291 nucleotides from the 5' end of λA2. Using these primers and *M. sexta* genomic DNA as template in a PCR, we obtained a 5.6-kb product (PCR-1) that overlapped the sequences of λE1 and λA2 and filled in the missing genomic DNA sequence. The entire region of the serpin gene-1 locus that was subcloned and sequenced is 21,536 bp long (Fig. 2).

**Expression of Serpin Gene-1 in Fat Body of *M. sexta* Appears to Be Negatively Regulated by Ecdysteroids (19). In computer searching to identify putative ecdysteroid receptor-binding sequences in serpin gene-1 we located a sequence beginning at position -327 (AGGTCAAGAACCT) which matches in 11 out of 13 positions with a consensus ecdysone receptor binding sequence (RGG/TTC/GANTGC/AC/ACTY) (20). Perhaps when ecdysteroid levels are elevated at periods such as the larval-prepupal stage, binding of an ecdysteroid to this sequence interferes with transcription of the serpin gene, resulting in the observed decrease in serpin-1 mRNA.

**Organization of the Serpin Gene-1—**Comparison of the genomic sequence with the sequence of cloned serpin-1 cDNAs indicated that serpin gene-1 is divided into 10 exons, with 12...
FIG. 2. Complete nucleotide sequence of *M. sexta* serpin gene-1. Nucleotides in the 5'-flanking region are assigned negative numbers. Nucleotide 1 is assigned based on primer extension data (17). Exon sequences are underlined. Untranslated regions of exons are double underlined. Amino acid sequences are listed below translated exons, using the one-letter code under the middle nucleotide of each codon.
Alternate forms of exon 9 (Figs. 1 and 2). From the transcription initiation site to the end of exon 10, the gene is 20,581 bp long. There is a clear difference in GC content between the exons (39% G+C) and the very A+T-rich introns (30% G+C). Exon 1 encodes the translation start site and the first six amino acid residues of the signal peptide. Exon 2 encodes the remainder of the signal peptide and the beginning of the mature protein sequence. Exons 3–8 encode the remainder of the serpin-1 sequence up to amino acid residue 336. Twelve alternate versions of exon 9 then occur within a stretch of approximately 11.6 kb. These are followed by exon 10, which encodes the 3′-untranslated sequence common to all of the serpin-1 cDNAs.

**Analysis of Intron Sequences**—We used the FASTA program (GCG) to search the GenBank DNA sequence data base with sequences of the introns from serpin gene-1 and found several high scoring matches with sequences from other *M. sexta* genes. A 28-nucleotide region within the first intron (between exons 1 and 2) beginning at position 853 was identical at 26 positions with a sequence in the upstream region of the *M. sexta* microvitellogenin gene (21). This alignment was done with the FASTA program (GCG). Positions identical in all four sequences are marked with an asterisk. Positions identical in three of the four sequences are marked with a plus sign.

![Fig. 3. Hybridization of *M. sexta* serpin-1B (alaserpin) cDNA clone to *M. sexta* genomic DNA. *M. sexta* genomic DNA (10 μg) was digested with restriction enzymes; the digested DNA was resolved by agarose gel electrophoresis, transferred to nylon membrane, and hybridized with 32P-labeled serpin-1B cDNA (10). The positions and sizes of DNA size markers are indicated at the right.](image)

Alternate exon use in the serpin gene-1. The lack of mismatches in this long inverted repeat suggests that there may be some selective pressure to preserve its structure and function.

**Sequence Analysis of the Exon 9 Region**—When we searched the sequence between exon 8 and exon 10 for regions that could encode the sequence Pro-Phe-Xaa-Phe, which is conserved in all of the serpin amino acid sequences (see Fig. 7A), we detected 12 open reading frames whose length and sequence are consistent with their identification as alternate versions of exon 9. These are named alphabetically A–K, and then Z. We named the most 3′ exon 9Z when we did not yet know the total number of exon 9 variants (8). We previously cloned cDNAs that contained regions corresponding with exon 9A, B, D, E, F, G, and Z (8). We also previously obtained cDNA clones for which we did not yet have genomic exon 9 sequence. These have now been located in the gene fragment obtained by PCR as exon 9J (previously named 9A), and exon 9K (previously exon 9k). For another cDNA clone with exon 9μ, differing from 9J(λ) only by a 6-bp deletion, we did not identify a comparable exon 9 in the genomic sequence. Thus, 9μ may be an allelic variant of 9J(λ).

To determine whether all of the exon 9 variants detected in the gene sequence are actually expressed in spliced serpin-1 mRNAs, we used a gene-specific primer and a vector-specific primer to amplify DNA from a *M. sexta* hemocyte cDNA library and cloned the resulting PCR fragments into plasmid vectors. In this way we obtained a large number of cDNA clones covering the 3′ part of exon 8 and all of exons 9 and 10. When these clones were sequenced we identified correctly spliced versions of all of the exon 9 variants, including exon 9C, H, and I, which were not obtained in previous library screening. These results indicate that all 12 variants of exon 9 can be correctly spliced and are present in serpin-1 mRNA populations from hemocytes.

![Fig. 4. Similarities of sequences in introns from serpin gene-1 with noncoding regions of other *M. sexta* genes. Panel A, alignment of a region from the first intron of serpin gene-1 with a sequence upstream from the transcription initiation site of the *M. sexta* microvitellogenin gene. This alignment was done with the FASTA program (GCG). Panel B, alignment of a region from the intron between exons 9I and 9J with sequences from an intron in *M. sexta* arylphorin gene A (22), an intron in the *M. sexta* ecdysone (ec1) hormone gene (23), and a sequence immediately upstream from the gene for *M. sexta* larval cuticle protein-14 (LCP-14) (24). The sequences from the ecdysone hormone and LCP-14 genes are shown as reverse complement of the plus strand DNA sequence. These sequences were aligned using the Pileup program (GCG). Positions identical in all four sequences are marked with an asterisk. Positions identical in three of the four sequences are marked with a plus sign.](image)
When we compared DNA sequences of exon 9 variants in the gene with the corresponding cDNAs, five minor discrepancies were identified: ATG (Met, at 9013) to TTG (Leu) in exon 9D; ATT (Ile, at 10089) to ATC (Ile) in exon 9E; GAT (Asp, at 10964) to GAC (Asp) in exon 9F; CTC (Leu, at 15646) to TTC (Phe) in exon 9J; TTT (Phe, at 17409–17410) to ACT (Thr) in exon 9K.

These differences may result from allelic variation. We analyzed the sequences at the junctions between the exons and introns of serpin gene-1 (Fig. 6). We speculate that differences in the intron sequences bordering the exon 9 variants compared with other exons in the gene might be involved in regulation of the mutually exclusive use of exon 9 variants.

There are some differences in the consensus sequences at the intron borders of the exon 9 variants compared with the consensus sequence for the other exons in the gene and with a consensus sequence derived from many Drosophila genes (25).

At position 2 4 at the 3' end of the introns, T is present in 9 out of 12 of the exon 9 variants. There are no A residues at this position in the exon 9 group. However, in the other group of exons, the consensus is A or T, with no G residues present at this position. The terminal branches of this tree group exon 9E with 9G and 9F with 9H. This suggests that the most recent rearrangement of the exon 9 region was the duplication of a pair of exons, encoded by the exon 9 variants includes the reactive site loop, which is the site for interaction of a serpin with the active site of a serine proteinase. Changes in the amino acid sequence of this region can alter a serpin’s selectivity for proteinase inhibition. It appears that multiple copies of exon 9 have arisen in serpin gene-1 through repeated exon 9 duplications. We have investigated the pathway by which such duplications might have occurred during evolution of M. sexta serpin gene-1.
**FIG. 7. Panel A**, alignment of the amino acid sequences encoded by the exon 9 variants of *M. sexta* serpin gene-1. The sequences were aligned using the Pileup program (GCG). The Ala residue experimentally determined to be at the P1 position of the scissile bond in the reactive site region of serpin-1B (32) is underlined. Positions with identical residues in all of the sequences are marked with an asterisk. Panel B, alignment of the nucleotide and flanking intron sequences of exon 9 variants. Complete exon 9 variants and flanking intron sequences were aligned using the Pileup program (GCG). The nucleotides near the 5' and 3' ends of the exon 9 variants are in bold, double underlined to show exon-intron junctions. The stop codon at the 3' end of each open reading frame is underlined.

9E and 9F to produce 9G and 9H (or vice versa). This hypothesis is supported by sequence similarity detected in intron sequences. A region of similarity was found in a 363-bp region near the 3' ends of introns H and H* adjacent to exons 9E and 9G. The Bestfit score for these aligned sequences is 9 standard deviations greater than the mean score for 50 alignments of the randomized sequences of the aligned regions. An even more significant similarity exists in alignment of 392-bp sequences near the 5' ends of introns H and H* adjacent to exons 9F and 9H (the Bestfit score is 36 standard deviations greater than the mean for 50 alignments of the randomized intron sequences).

**DISCUSSION**

We have isolated DNA clones including the locus for *M. sexta* serpin gene-1 and have determined the nucleotide sequence of a 21.5-kb region that includes the serpin gene-1. The gene is composed of 10 exons, with 12 alternate versions of exon 9. Analysis of intron positions in many genes from the serpin superfamily has shown that there are a large number of sites at which introns are present, and it is likely that new introns have been acquired numerous times during divergence of the superfamily (26). *M. sexta* serpin gene-1 has introns at a few positions identical with some vertebrate serpin genes. The intron between exons 5 and 6 is at the same position as introns in genes for human α-antichymotrypsin, α-1-proteinase inhibitor, protein C inhibitor, antithrombin III, C1 inhibitor, leuserin, α2-antiplasmin, angiotensinogen, and mouse serpin J6. This position must therefore be an ancient location for an intron in these genes, prior to the evolutionary divergence of the invertebrates and vertebrates, and that this intron may have been lost from other vertebrate serpin genes.

The intron between exons 8 and 9 in *M. sexta* serpin gene-1 is most intriguing because its presence allows for the alternate exon splicing to produce multiple serpin mRNAs that encode proteins with a constant protein framework and variable reactive site sequences. Among other serpin genes that have been sequenced, an intron at this position has been identified only in the genes for human and rat plasminogen activator inhibitor-1 (27, 28). Although no alternate exons have been observed in these genes, the potential exists for alternate exon use in other serpin genes that contain an intron at this position.

Serpin gene duplication and sequence divergence have resulted in the presence of families of serpin genes in mammals such as humans, mice, and cattle (29–31). Sequence divergence in these genes is greatest in the region that encodes the reactive site, resulting in rapid evolution of serpins with new inhibitor selectivities (4, 30). Evolution of the capacity to produce serpins with a variety of reactive site sequences has followed a different route in *M. sexta* serpin gene-1. Instead of duplication of the entire gene, only the exon encoding the reactive site loop has been duplicated. Evolutionary sequence divergence of these exons has led to the ability to synthesize serpins with 12 different reactive site sequences from a single gene through use of mutually exclusive alternate exon splicing. Similar alternate exon use probably occurs in another lepidopteran insect, the silkworm *B. mori* (7). It will be of interest to determine how widespread this type of serpin gene structure may be in other insects and arthropods.

**FIG. 8. Phylogenetic tree of the 12 exon 9 sequences, derived from the nucleotide sequence alignment shown in Fig. 7B.** The tree was derived using the Distances and Growtree programs (GCG).
Manduca sexta Serpin Gene-1

10 (8), we have found that serpin-1 mRNAs contain only a single exon 9 sequence. Differences in the sequences at exon 9-intron borders compared with other exons in the gene (Fig. 6) might be signals for regulation of the splicing process.

From the analysis of the alignments of nucleic acid sequences of the exon 9 variants and alignments of the amino acid sequences they encode (Figs. 7 and 8) we propose that an ancestor of serpin gene-1 contained a single exon 9 that was duplicated. These may have been the precursors of exons 9A and 9Z. Further duplication events led to exons 9B and 9K and insertion of an additional codon for proline at the carboxyl terminus. From an ancestor of 9K diverged the remaining exons, all with a four-codon insertion at the carboxyl-terminal end of the reactive site loop. The tree then forms two branches, one with exon 9C and another with 9J as the last common ancestor. The most recent duplications involve exons 9D, 9E, 9F, 9G, 9H, and 9I, all near the center of the exon 9 region, with duplication of 9E and 9F as a pair to produce 9G and 9H.

Of course this speculation about evolution is based on the observable structure of the gene from a single specimen of a contemporary M. sexta. The actual path by which the exon 9 variants arose may actually have been more complex. It is likely that duplication of these exons has occurred through unequal crossing over. At each such event one daughter chromosome would have more copies of exon 9, and the other would have fewer. Furthermore, once multiple copies of the exon were present, several copies could be gained or lost at each instance of unequal crossing over. It seems likely that change in the number of copies of exon 9 in serpin gene-1 is a continuing process and that individuals from different populations could have a serpin gene-1 structure distinct from the one we have characterized. It will be necessary to examine the structure of serpin gene-1 from different M. sexta strains and from other Manduca species to understand more fully the evolution and population genetics of this gene.

Why does M. sexta need so many different proteinase inhibitors? The physiological roles of serpins in insects have not yet been established. We have expressed the serpin-1 variants as recombinant proteins and found proteinase inhibitor activity for 11 of the 12 reactive sites. The individual variants can inhibit enzymes with chymotrypsin-, elastase-, or trypsin-like specificities. Serpins in insect hemolymph might inhibit proteinases produced by pathogenic microorganisms. This role has been postulated for families of serpins in mice (2, 4). Another potential function for these inhibitors is regulation of endogenous serine proteinases in hemolymph. Proteinase zymogens are present in plasma, and others may be released from hemocytes in inflammation-like responses. We have cloned cDNAs for five different serine proteinases expressed by M. sexta hemocytes, and we have identified biochemically several different proteinase activities in hemolymph plasma. Some of the serine proteinases in insect hemolymph are involved in a cascade that leads to activation of hemolymph prophenoloxidase (6). Phenoloxidase activation occurs in response to wounding and bacterial or fungal infection. The quinones and melanin resulting from phenoloxidase activity appear to be components of a protective response to microbial infection in insects. Regulating this pathway may be an important role of some of the serpin-1 variants. Thus, the inhibitors produced from M. sexta serpin gene-1 may participate in several physiological functions related to wound healing and antimicrobial defense. It is also quite likely that these serpins inhibit endogenous proteinases involved in processes in insect physiology which have not yet been discovered.

Acknowledgments—We thank Dr. Michael Wells, University of Arizona, for the use of laboratory facilities for the initial phases of this work. We thank Drs. Robin Denell, Karl Kramer, Subbaratnam Muthukrishnan, and Gerald Reec for helpful comments on the manuscript.

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