Alternative Splicing of the Primary Transcript Generates Heterogeneity within the Products of the Gene for Bombyx mori Chitinase*

Babiker M. A. Abdel-Banat and Daizo Koga‡

From the Laboratory of Biochemistry, Department of Biological Science, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan

The gene of chitinase in the silkworm, Bombyx mori, generates four mRNA products by alternative splicing. Nucleotide sequences of the entire gene for chitinase and respective cDNAs demonstrate that the pre-mRNA undergoes alternative splicing at both the 5′ and 3′ regions. At the 5′ region, the pre-mRNA experienced differential splicing through two alternative 5′-intron consensus splicing sites. These products differ in the last amino acid of the signal peptide and the first amino acid of the mature N-terminal sequences: one with Cys20-Ala21 and the other with Ser20-Asp21. The product with Cys20-Ala21 residues is one amino acid larger than the other with Ser20-Asp21. At the 3′ region the pre-mRNA of the chitinase gene undergoes alternative splicing in three different fashions. It is spliced either through retaining or excluding the upstream 121-bp direct repeat found at the 3′ region of the coding sequences or through retaining or excluding of an insertion of 9 bp in a combinatorial manner. Retention or exclusion of the upstream 121-bp direct repeat results in a protein with a deduced amino acid sequence similar in size to the one retaining both direct repeats. However, exclusion of the insert of the 9 bp from the mRNA results in a protein with 22 extra amino acids. All of the mRNA products appear to be generated from a single gene as demonstrated by testing the 3′-intron-repeated region of the genomic DNA and variant chitinase mRNA products. B. mori chitinase expression in the fifth instar larvae epidermal tissues appears to be developmentally regulated, but the phenomenon of alternative splicing of the pre-mRNA is not stage-dependent. Furthermore, the four mRNA products showed chitinase activity when expressed in Escherichia coli, which demonstrates the role of the alternative splicing process in generating multiple isoforms of the silkworm’s chitinase.

There are many sources of chitinases, including bacteria, fungi, plants, marine organisms, insects, and mammals (1). The role of these enzymes differs among the organisms. In insects, they degrade the biopolymer chitin, which is found in the exoskeleton and gut lining. Insect chitinases are induced by ecdysteroids at the time of molting and metamorphosis of the larvae to degrade most of the older chitin (2, 3). Chitinase expression in the insects’ exoskeletons and the guts normally occurs only during molting, where the chitin of the integument and the peritrophic membrane is presumably degraded (4). Expression of the chitinase at a precise timing in insects demonstrates its fundamental role in the process of ecdysis. To elucidate the functions of the chitinases, the genes for these enzymes have also been isolated and characterized from many organisms, including mammals, insects, fungi, nematodes, and bacteria (5–12). One of the structural features observed in chitinases is a multidomain architecture that includes catalytic domains, a cysteine rich chitin-binding domain, and a serine/threonine-rich domain that is glycosylated (13). The conserved motifs in most of the insects’ chitinase genes reside in the central domains, which include the two regions implicated in catalysis (9). Differences occur in the cysteine-rich carboxyl domain and the proline/glutamate-serine/threonine-rich domain that is presumed to increase the susceptibility to proteolysis by a calcium-dependent protease (14, 15). The silkworm, Bombyx mori, chitinases are well studied in our laboratory at both the enzymological and DNA levels (9, 16, 17).

We have previously reported a complete exploration of the gene for the chitinase from B. mori (9). The gene’s structural organization and functional motifs are consistent with the multidomain architecture of the proteins (13). The organizations of its functional domains are identical to the related gene from Manduca sexta (18). Hence, the genes for cuticular chitinases may be conserved among insect species (9). The coding regions of the genomic clone for the gene of B. mori chitinase have unique features both at exon 1/intron 1 splicing junctions and the 3′-end of the gene’s coding regions (Fig. 1). First, the invariant nucleotides, GT, at the 5′ boundary of intron 1 are repeated in near proximity, so we suggested a differential splicing of the pre-mRNA (9). Another feature of this gene includes the incorporation of an additional sequence of 9 bp at the 3′-end of the coding regions (Fig. 1). This sequence is absent from a chitinase cDNA clone studied earlier (7). The open reading frame (ORF) is interrupted by the inclusion of the 9 bp due to the stop codon within these nucleotides. Therefore, the protein encoded by our chitinase gene is 22 amino acids smaller in size. Furthermore, another cDNA encoding a putative chitinase of B. mori has been reported recently (8). There are some discrepancies between these two sequences of the chitinase cDNAs (7, 8). They differ in the first amino acid of the mature N-terminal

* This work was supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AB052914 and AF455139.

‡ To whom correspondence should be addressed. Tel.: 81-83-933-5862; Fax: 81-83-933-5820; E-mail: koga@agr.yamaguchi-u.ac.jp

1 The abbreviations used are: ORF, open reading frame; RT, reverse transcription; IPTG, isopropyl β-D-thiogalactopyranoside; CAPS, 3-(cyclohexylamino)propanesulfonic acid; DTT, dithiothreitol; GST, glutathione S-transferase.

Received for publication, December 28, 2001, and in revised form, April 17, 2002
Published, JBC Papers in Press, June 3, 2002, DOI 10.1074/jbc.M112422200
region and in the C-terminal coding region. In addition, there are direct repeats of 112 bp in the former cDNA sequence (7), but these were not found in the latter study (8). Our previous studies on B. mori chitinases revealed that the first amino acid of the mature N-terminal sequences is alanine (16). These results are supported by deduced amino acid sequences of the mature N-terminal sequences is alanine (16). These results are supported by deduced amino acid sequences of the mature N-terminal sequences is alanine (16).

Alternative pre-mRNA splicing is an important mechanism in gene regulation and can include the use of alternative 5′- and/or 3′-splice sites, exon skipping, mutual exon exclusion, and/or intron retention (19–21). Although intron retention is common in viral pre-mRNAs, it appears to be a relatively rare form of alternative splicing in vertebrates (22). It is believed that intron-containing mRNAs normally are prevented from being transported to the cytoplasm due to the formation of the spliceosome complex, which commits the pre-mRNA to the splicing pathway (23–25). However, there are examples of alternative intron retention in vertebrates that result in novel proteins due to translation of the intron sequences (26). However, there are examples of alternative intron retention in vertebrates that result in novel proteins due to translation of the intron sequences (26).

![](image)

**FIG. 1. Structure of B. mori chitinase gene.** Exons (E1–E10) are shown as hatched rectangles and introns (I1–I9) as open rectangles below the size scale. Part of the nucleotide sequence spanning the area between exons 1 and 2 and the sequences of the 121-bp direct repeats in exon 10 are presented under the gene’s structural scheme. Exon sequences are shown in capital letters, and intron sequences are in small letters. The alternate 5′-intron 1 splicing site is indicated by the shill mark (ⅰ). The underlined nucleotides code for the underlined amino acid, cysteine, while the boxed nucleotides code for the boxed amino acid, alanine. The sequences of the 121-bp direct repeats in exon 10 that are indicated by white arrows within the structural scheme are underscored with inverted arrows, and the 9-bp insert DNA is boxed. The stop codon, TAA, is in boldface letters.

**EXPERIMENTAL PROCEDURES**

**Preparation of Total RNA from B. mori Cuticular Tissues—**The silk-worms, B. mori strain (Kinshu × Showa), were reared on mulberry leaves at a room temperature of 27 °C with a photoperiod of 13 h:11 h light/dark. The epidermal tissues of the integument were collected from the fifth instar larvae on the day when spinning behavior started (SP-0) and 1, 2, 2.5, and 3 days later (SP-1, SP-2, SP-2.5, and SP-3, respectively). The tissues were prepared as described previously (9). Total RNAs were isolated from the frozen tissues using the RNeasy Midi kit (Qiagen, Inc.) according to its protocol.

**Chitinase cDNA Synthesis and Amplification—**Chitinase cDNAs were synthesized using the total RNAs obtained from the five different stages with the Ready-To-Go RT-PCR kit (Amersham Biosciences) according to the instructions of the manufacturer. The first-strand cDNA synthesis was done on 2 μg of total RNA using reverse transcriptase (FPLCpure) and the oligo(dT) primer, pdT17(17–19) (Amersham Biosciences). To amplify the cDNAs, each PCR was primed with two gene-specific primers designed from the B. mori genomic clone for the chitinase gene (9). The primers used in these amplifications were:

- A sense primer designed at the 5′-end of the gene (BMB XbaI 1 (5′-GCC GTTACAG AAAAAAT CGGAGCATTTTGC-3′)). The underlined sequence is the site for XhoI and three antisense primers. One of them starts from the 9-bp insert DNA, which is found in the genomic clone, and contains a stop codon. Its position is at nucleotides 13,828–13,847 of the genomic clone for the chitinase gene (9) (BMB SacI 8 (5′-CCCGAGCT TCTTACGAAATCCAGGGTATTTG-3′)). The second antisense primer was designed at nucleotide positions 14,534–14,553 of the genomic clone (9) and the underlined sequence is the site for SacI and three antisense primers. One of them starts from the 9-bp insert DNA, which is found in the genomic clone, and contains a stop codon. Its position is at nucleotides 13,828–13,847 of the genomic clone for the chitinase gene (9) (BMB SacI 8 (5′-CCCGAGCT TCTTACGAAATCCAGGGTATTTG-3′)). The second antisense primer was designed at nucleotide positions 14,534–14,553 of the genomic clone (9) and named BMB SacI 10 (5′-CCCGAGCT TCTTACGAAATCCAGGGTATTTG-3′). The underlined sequence in both primers design the SacI recognition site within the primers. The third antisense primer was synthesized at the 3′-end of the gene with the XhoI recognition site (BMb14 (5′-CCCGAGCT TCTTACGAAATCCAGGGTATTTG-3′)). The underlined sequence in both primers design the SacI recognition site within the primers. The third antisense primer was synthesized at the 3′-end of the gene with the XhoI recognition site (BMb14 (5′-CCCGAGCT TCTTACGAAATCCAGGGTATTTG-3′)). Amplification reactions (50 μl) contained 7 μl of first-strand cDNAs, 5 μl of 10× Ex Taq buffer, 4 μl of dNTP mixture (2.5 mM each), 1 μl of sense and antisense primers (20 pmol each), 31.8 μl of sterile distilled water, and 0.2 μl of TaKaRa Ex Taq DNA polymerase (5 units/μl) (Takara Shuzo Co., Ltd.). Cycling parameters were set as follows: one cycle of preheating at 94 °C for 3 min and 30–40 cycles of each of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, and ex-
tension at 72 °C for 3 min. A final post-extension cycle was performed at 72 °C for 7 min.

**Southern Blotting and Hybridization**—A cDNA clone of 1758 base pairs in length previously synthesized and detected containing an insert of 9 bp by sequence analysis was used as probe. It was prepared from 2 mg of the fifth instar larval RNA using the primer BMB Xho I 1, and an antisense primer, BM5 (5'-GGCGAATTCCTTAC-GAACATTCCGTGCT-3'), which was designed from the genomic DNA sequence (9) at nucleotide positions 13,950–13,968. The probe was labeled using the Gene Images Random Prime Labeling Module (Amersham Biosciences). Electrophoresis of the target DNAs and their transfer onto the membrane was carried out as described previously (9). The DNAs were allowed to transfer overnight onto the Hybond-N+ membrane and then fixed to the membrane by heating at 80 °C for 2 h.

Hybridization buffer was composed of 5× SSC containing 0.1% SDS, 5% dextran sulfate, and 20-fold dilution of liquid block. Hybridization was performed overnight at 65 °C with gentle agitation. The blots were washed for 15 min in the first stringency wash solution (1× SSC containing 0.1% SDS), which was pre-heated to 65 °C. A further stringency wash was carried out at the same temperature in a pre-heated solution of 0.5× SSC containing 0.1% SDS. Blocking of the blots, antibody reaction, removal of the unbound conjugate, signal generation, and detection were performed according to the manufacturer's protocol. 10-min exposures were made of the membrane using Fuji x-ray film, and the film was developed.

**Expression of Chitinase in Fifth Instar Larvae**—Total RNA was extracted from five stages of the fifth instar larvae as described above. For Northern hybridization analysis, the total RNAs (10 μg from each of SP-0, SP-1, SP-2, SP-2.5, and SP-3) were denatured in formaldehyde, been soaked in 0.4M NaOH and briefly rinsed in 2

---

**Fig. 2.** PCR amplification of *B. mori* chitinase cDNA from SP-3 larvae and Southern blotting. A, agarose gel electrophoresis of the RT-PCR products. The products were separated on 1.2% agarose gel and stained with ethidium bromide. Lanes 1 and 2, amplified products with the primers BMB Xho I 1 and BMB Sac I 10; lanes 3 and 4, amplified products with the primers BMB Xho I 1 and BMB Sac 18; lanes 5 and 6, amplified products with the primers BMB Xho I 1 and Bmb 14. The DNA molecular weight markers, pHY and λ-Bst PI digest, were applied in the border lanes. Relative positions of the primers are drawn to scale below the panel. B, Southern blotting and hybridization of the cloned cDNAs. Recombinant cDNAs, amplified with the primers BMB Xho I 1 and Bmb14 from individual bacterial colonies were subjected to electrophoresis on 1.2% agarose gel and probed with a fluorescein-labeled cDNA clone whose sequence was identified earlier (see "Experimental Procedures"). Lanes 1–7, cDNAs from seven different recombinant bacterial colonies hybridized to the probe; lane P, the probe used for hybridization applied as positive control. Relative positions of the primers used for amplification of the probe are drawn to scale below the panel.
FIG. 3. Nucleotide sequence and the deduced amino acids of *B. mori* chitinase cDNA, clone SP-3/1 (1–14). The deduced amino acid sequence is shown on the top of the nucleotide sequence. The upward arrowhead indicates the position of the differentially spliced triplet (GTG). The 9-bp insert DNA is boxed, and the stop codon within the insert, TAA, is shown in boldface letters. The sequences of the direct repeats are underscored with dotted lines with arrowheads at the ends. The putative chitinase signal peptide sequence is underlined. The first residue of the mature N-terminal amino acids, aspartate, is also boxed. The sequence has been deposited in the GenBank® data base (accession number AB052914).
samples were heated at 70 °C for 3 min and loaded into the gel. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250. Myosin (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66.4 kDa), ovalbumin (52.4 kDa), carbonic anhydrase (34.9 kDa), soybean trypsin inhibitor (29.1 kDa), lysozyme (20.7 kDa), and albumin (66.4 kDa), ovalbumin (52.4 kDa), carbonic anhydrase (34.9 kDa). The other subcloned fragments (1637 and 2346 bp) were tested only by PCR amplifications, because a common sense primer was used for all PCRs, and therefore the amplified products were expected to be from the same target gene. The recombinant pBluescripts with the different clones from the SP-3 larvae were sequenced, and the results from six sequencing rounds revealed that all clones sequenced so far with homologous sequences differed only in their corresponding sizes (Fig. 3). Interestingly, all cDNAs sequenced carried the insert of 9 bp at the same position, which includes a stop codon, TAA. This 9-bp insertion mediates a direct repeated sequence of 112 bp. If the nucleotides of this insertion were summed up to the upstream 112 nucleotides, the direct repeats will be of 121 nucleotides (Fig. 1). The complete sequence of the cDNA had an ORF of 1629 nucleotides, encoding a protein of 543 amino acid residues (Fig. 3) that has a calculated molecular mass of 60,979 Da.

Identification of Chitinase cDNA Clones Generated by Alternative Splicing of Pre-mRNA—Analysis of the B. mori genomic clone for the chitinase gene (9) brought to light the possibility of alternative splicing of the pre-mRNA to yield variant mature mRNAs (Table I and Fig. 1). To prove the occurrence of this phenomenon in B. mori chitinase gene, many other cDNAs of different sizes were synthesized and analyzed from the stages SP-0 through SP-2.5 of the fifth instar larvae. Sequence analysis of the cDNA clones revealed that alternative splicing of the primary transcript yielded variant mature mRNAs of the B. mori chitinase gene (Figs. 3 and 4). The relative distributions of the products of the alternatively spliced mRNAs are summarized in Table II. The mRNAs of the four clones (SP-1/(1–10)2, SP-2/(1–10)1, SP-2/(1–10)2, and SP-2/(1–14)) were found to retain the three nucleotides at the junction between exon 1 and intron 1, whereas those of the other seven clones analyzed (SP-1/(1–10)1, SP-1/(1–14)1, SP-2/(1–8), SP-2/(1–10), SP-3/(1–8), SP-3/(1–10), and SP-3/(1–14)) were lacking these nucleotides. The mRNAs of the six clones were found to retain the 9 bp at the 3’-end of the gene, whereas those of the other two clones excluded these nucleotides. Furthermore, in three clones (SP-1/(1–10)2, SP-2/(1–10)2, and SP-2/(1–14)), the upstream 121-bp direct repeat, including the 9 bp, underwent alternative splicing to become part of intron number 9 of the chitinase gene (Table II and Fig. 5, A and B). Retention and exclusion of these sequences appears to be independent of the stage of the fifth instar (Table II).
FIG. 4. Nucleotide sequence and the deduced amino acids of *B. mori* chitinase cDNA, clone SP-2/(1–10). The triplet (GTG) that is retained in this clone is **boldfaced** and **underlined**. The **downward arrowhead** indicates the insertion site for the excluded 9 bp. The sequences of the 112-bp direct repeats are **underscored** with **inverted arrows**. The sequence identical to the excluded 9 bp is **boxed**. The amino acid, alanine, which is introduced due to the inclusion of the triplet (GTG), is **boxed**. The sequence has been deposited in the GenBank™ data base (accession number AF455139).
Effect of Alternative Splicing on Generating Heterogeneity within the Gene Products for Chitinase—To investigate the effect of alternative splicing on generating variations within the products of the primary transcript of *B. mori* chitinase gene, the deduced amino acid sequences of *B. mori* chitinase cDNAs elaborated from the sequenced clones were aligned (Fig. 6). Sequence alignment revealed that the encoded chitinase from the different clones showed exactly the same amino acid residues. However, retention of the triplet (GTG) at the exon 1 and intron 1 splicing boundary changes the last amino acid residue of the putative chitinase signal peptide from serine to cysteine and introduces additional amino acid, alanine, at the mature N-terminal sequence. This alanine is consistent with the chitinase N-terminal sequence (ADSRARIVCYFSNWAV-YRPG) (16). Exclusion of the triplet results in a mature N-terminal sequence. This alanine is consistent with the presence of a large intron separating the repeated sequences.

Table I

| Stage/clone | Exon1/Exon2 | Exon10 |
|-------------|-------------|--------|
| SP-1/1–10| -GTG | +9 |
| SP-1/1–14| -GTG | +9 |
| SP-2.5/1–10| -GTG | +9 |
| SP-3/1–8| -GTG | +9 |
| SP-2/1–10| -GTG | +9 |
| SP-3/1–14| -GTG | +9 |
| SP-1/1–10/2| +GTG | -121 |
| SP-2/1–10/2| +GTG | -121 |
| SP-2/1–14| +GTG | -121 |
| SP-2/1–10/1| +GTG | -9 |
| SP-2.5/1–8| +GTG | -9 |

* – GTG and + GTG indicate the cDNA clones without (−) or with (+) the GTG triplet at the 5’-region. +9 and −9 indicate the cDNA clones with (+) or without (−) the 9-bp insert DNA at the end of the upstream 121-bp direct repeat. −121 represents exclusion of the upstream 112-bp direct repeat together with the 9-bp insert DNA from the cDNA clone.

Confirmation of the Role of Pre-mRNA Splicing—To verify that the cDNAs described above were produced by alternative splicing and were not transcribed from additional gene sequences elsewhere in the genome, the same primer set was used in PCR with genomic DNA as the template. The expected size of genomic PCR product for the primer set was obtained. The DNA fragments amplified from SP-0 through SP-3 genomic DNAs were typically the same size as expected (Fig. 7A). The same primer set was used in PCR with cDNA clones that exhibited differential splicing of the upstream 121-bp direct repeat (Fig. 7B). The results indicated that the variant cDNA clones investigated were the products of a single gene, which in turn supported the notion that the copy number of the *B. mori* chitinase gene was 1 per haploid genome (8). In addition, the results are not consistent with the presence of a large intron separating the repeated sequences.

Developmental Changes in Chitinase mRNA Levels—Chitinase cDNAs were synthesized from the fifth-instar larval RNA on the day of spinning behavior through 3 days later (SP-0 through SP-3). To assess the changes in the levels of chitinase mRNA in these epidermal tissues, total RNAs from the respective tissues were analyzed by Northern blotting. The blots were probed with the fragment BMB XbaI 1 − BMB ScaI 10 (Fig. 2A). Chitinase mRNA was undetected by Northern blotting.

![Fig. 5. B. mori chitinase gene structure and the alternatively spliced products. A, the schematic diagram at the top represents the chitinase gene structure (9). The hatched rectangles represent the exons (E1–E10) and the introns (introns 1–9) are marked by the open rectangles. The triplet (GTG) is shown in capital letters within intron 1, and the second alternative splicing position is indicated by the shill mark (l). The direct repeats 112 + 9 and 121 bp are indicated by the solid rectangles, and the white arrows show their direction. The open rectangle between the direct repeats represents the position of the 9 bp. The two diagrams below illustrate the coding regions of the alternatively spliced mature transcripts. The cDNA clones excluding and retaining the triplet (GTG) are indicated by −Ala21 and +Ala21, respectively. 112 + 9 and 121 bp under the coding regions' diagram indicate that the mature transcripts retained both of the direct repeats and the 9 bp, whereas 112 and 121 bp indicate that only the 9-bp insert was spliced in a combinatorial fashion. B, the top diagram is similar to that in A, illustrating that the 9-bp and the upstream 112-bp direct repeat were spliced as part of intron 9. The product of the primary transcript is shown at the bottom. C, the structure of a previously reported cDNA clone (8). The clone retained the triplet (GTG) at the 5’ region as shown by +Ala21, but excluded the upstream 121-bp direct repeat. D, the structure of another chitinase cDNA clone (7). This clone excluded both the triplet (GTG) at the 5’ region as indicated by −Ala21 and the 9-bp insert, which is at the end of the upstream 121-bp direct repeat. However, the clone retained the 112-bp direct repeats as shown by 112 and 121 bp below the diagram. The DNA fragments amplified from SP-0 through SP-3 genomic DNAs were typically the same size as expected (Fig. 7A). The same primer set was used in PCR with cDNA clones that exhibited differential splicing of the upstream 121-bp direct repeat (Fig. 7B). The results indicated that the variant cDNA clones investigated were the products of a single gene, which in turn supported the notion that the copy number of the *B. mori* chitinase gene was 1 per haploid genome (8). The results are not consistent with the presence of a large intron separating the repeated sequences. Developmental Changes in Chitinase mRNA Levels—Chitinase cDNAs were synthesized from the fifth-instar larval RNA on the day of spinning behavior through 3 days later (SP-0 through SP-3). To assess the changes in the levels of chitinase mRNA in these epidermal tissues, total RNAs from the respective tissues were analyzed by Northern blotting. The blots were probed with the fragment BMB XbaI 1 − BMB ScaI 10 (Fig. 2A). Chitinase mRNA was undetected by Northern blotting.
on the day when fifth instar larvae initiated spinning behavior. However, it was present at very low levels 1 day later, and its levels increased sharply on the second day and gradually onward (Fig. 8A). To test the level of the expressed chitinase mRNA that affect detection of the mature chitinase transcripts by RT-PCR, cDNAs were synthesized using half the amount (5 μg) of the total RNAs used for Northern blotting as templates for the first-strand cDNA synthesis. As shown in Fig. 8B, all cDNAs plotted were amplified with the B. mori chitinase gene-specific primers, BMB XbaI1 and BMB SacI10, and the respective first-strand reactions as templates for PCR. The results showed expression of the chitinase mRNAs in all stages of the fifth instar larvae investigated, which means the chitinase in B. mori is gradually expressed in the larval epidermal tissues from the day when the fifth instar larvae commenced with spinning behavior.

Protein Expression and Detection of the Chitinase Activity—

The four cDNA constructs generated by alternative pre-mRNA splicing of the silkworm chitinase gene were expressed in E. coli strain Tuner(DE3)pLacI. After induction with IPTG, the total cell proteins from induced and uninduced transformed cell were subjected to SDS-PAGE. Intense bands of about 60 kDa were observed (Fig. 9A). Chitinase activity was assayed with glycol chitin-containing gel (32). The recombinant proteins produced active bands on the gel containing the substrate (Fig. 9B).

**DISCUSSION**

Sequence alignment of the coding regions in the B. mori genomic clone for the gene of chitinase (9) with the chitinase cDNA (7) revealed the correspondence of the two clones. However, the genomic clone incorporated an insert of 9 bp (5'-GTTCGTAAG-3') at the C-terminal end of the gene, which was not reported in the chitinase cDNA (7). The fifth nucleotide of the 9 bp was found to be A instead of G in some of the cDNA clones, which may be due to sequencing ambiguities. It appears that these nucleotides increase the size of the encoded protein by three amino acids. In contrast, the protein encoded by this clone was found to be of smaller size when compared with the one deduced from the chitinase cDNA (7) because of the presence of a stop codon within the 9-bp insert DNA (Fig. 1).
clone of the *B. mori* chitinase gene suggested both cases to be true. To clarify the ambiguous results observed at the N-terminal region of the chitinase gene, to verify the existence of the 9-bp insert DNA in the mRNA, and to determine at which level these sequence differences are regulated, cDNA clones from the same insect species were synthesized at different stages of the fifth instar larvae and analyzed. Previously, we proposed that these discrepancies might be due to the insects’ strains used in the different studies or otherwise regulated at the level of pre-mRNA splicing (9).

There are many genes reported to exhibit alternative RNA splicing in various organisms (34). Among insects, the tobacco hornworm, *M. sexta*, serpin gene produces 12 different serine proteinase inhibitors (serpins) through alternative pre-mRNA splicing (19, 21). This splicing process generates inhibitor diversity and potentially regulates a variety of proteinases, using the same protein framework joined to different reactive site region cassettes (19). There are also about ten genes in *Drosophila melanogaster* found to exhibit different patterns of alternative splicing. The class I glutathione *S*-transferase (*GST*) gene family was also found to undergo alternative splicing in *Anopheles gambiæ* (35) and *Anopheles dirus* (36). In both species the GST gene contains six exons for four mature GST transcripts, which share exons 1 and 2 but vary between four different exon 3 sequences (exons 3A–3D) (36). The sericin 1 primary transcript of *B. mori* is differentially spliced via a tissue- and developmentally regulated process (37). The structure of the sericin 1 gene is characterized by the presence of a large central alternative exon, which encodes an internally repetitive sequence (37). The serpin gene-1 from *M. sexta* is characterized by the presence of 12 alternate forms of exon 9. The splicing pathway apparently allows inclusion of only one exon 9 per molecule of a mature serpin-1 mRNA (21).

In this study we demonstrated that the *B. mori* chitinase gene exhibits four patterns of alternative pre-mRNA splicing: one at the 5' region and three at the 3' region (Figs. 5 and 6). In some of the cDNA clones amplified (SP-1/1–10/2, SP-2/1–10/1, SP-2/1–10/2, and SP-2/1–14)), the 3' border of exon 1 was found to be three nucleotides downstream (GTG/gtgagt), giving a more conventional intron entry site, gtagt (37) (Tables I and II and Fig. 1). In this case the gene undergoes differential splicing through two alternative 5'-intron splicing consensus sites at the boundary between exon 1 and intron 1.

**FIG. 7.** PCR amplification of the genomic DNA and the chitinase cDNA clones. A, genomic DNAs from SP-0 (lane 1), SP-1 (lanes 2 and 3), SP-2 (lanes 4 and 5), SP-2.5 (lanes 6 and 7), and SP-3 (lanes 8 and 9) were used as templates for PCR with the primers BMB9 and BMB SacI 10. B, the cDNA clones SP-1/1–10/1, SP-2/1–10/2, SP-3/1–10/1, SP-1/1–10/2, SP-2/1–10/2, and SP-2/1–14) were used as templates for PCR in lanes 1, 2, 3, 4, 5, and 6, respectively. The primer set for all PCR amplifications was BMB9/BMB SacI 10. Lanes 1–3 contain the products that excluded the upstream 121-bp direct repeat, while lanes 4–6 contain the products that retained both direct repeats. Lanes M represent the DNA molecular weight marker (pH3). The relative positions of the primers used for the amplifications are drawn to scale below the panels.

**FIG. 8.** Northern blotting and expression assessment of chitinase mRNA. A, Northern blot analysis of chitinase mRNA. Total RNAs (10 μg) were collected from epidermal tissues of the fifth instar larvae on the day of the spinning behavior (SP-0) through 3 days later (SP-1, SP-2, SP-2.5, and SP-3). Blotted onto a Hybond-N membrane, and hybridized with fluorescein-labeled cDNA clone as described under "Experimental Procedures." B, PCR amplification of cDNAs from the fifth instar larval epidermal tissues (SP-0 through SP-3). Five μg of total RNA from each stage was used for the synthesis of the first-strand cDNA. The cDNAs were synthesized with the products of the first-strand reactions as templates and the primers, BMB XbaI and BMB SacI 10. The products were subjected to 1.2% agarose gel electrophoresis and stained with ethidium bromide. The position of the corresponding bands is indicated by the arrowhead. Lane M, DNA molecular weight marker (pH3).
Alternative Pre-mRNA Splicing of B. mori Chitinase

not interrupt the ORF of the corresponding mature transcripts. It is noteworthy that the 5′ region in the B. mori chitinase gene (9) is very unique compared with its closely related gene from M. sexta (18), both in sequence and structural motifs, because there is only one functional 5′-intron splicing consensus sequences in the junction between exon 1 and intron 1 in the M. sexta gene for chitinase (Table I). This might be attributed to species differences.

Another striking feature of the B. mori chitinase gene is its incorporation of 9 bp at the 3′ region (9). This insertion mediates direct repeats of 112 bp. However, if the 9-bp insert DNA is summed up to the upstream 112 bp, the direct repeats will be 121 bp (Fig. 5). There are two types of splicing patterns in this region of the gene. A splicing occurs via exclusion of the upstream 121-bp direct repeat in some of the mature transcripts (SP-1/(1–10)2, SP-2/(1–10)2, and SP-2/(1–14)) (Table II), because the 3′-end of the 9-bp insert DNA serves as an alternative intron splicing site. The stop codon in the product of this splicing pattern was shifted to the downstream 121-bp direct repeat. The proteins encoded by either inclusion or exclusion of the upstream direct repeat will be of similar size in terms of their amino acids (Fig. 6).

To verify that the cDNAs described were produced by splicing and were not transcribed from additional gene sequences elsewhere in the genome, the same primer set was used in PCR with genomic DNA as the template to amplify the 3′ region around the direct repeats. Nucleic acids that differ by 121 bp are detectable on agarose gel. The DNA fragments amplified from SP-0 through SP-3 genomic DNAs were typically the same size as expected (Fig. 7A). The same primer set was used in PCR with cDNA clones that exhibited differential splicing of the upstream 121-bp direct repeat as the templates (Fig. 7B). The results clearly indicated that the variant mature transcripts investigated are the products of a single gene, which in turn supports the concept that the copy number of B. mori chitinase gene is 1 per haploid genome (8). In addition, these results are inconsistent with the possibility that the nucleotides of the 9 bp are part of a large intron separating the repeated sequences. Moreover, the 9-bp insert DNA was also determined by sequencing to be alternatively spliced (Figs. 4 and 5). The mechanism by which the gene undergoes splicing of the 9 bp is not clear. However, the 9 bp may be spliced in a combinatorial fashion, because, in this pattern of splicing, the alternatively spliced genes contain entire exons that are individually included or excluded from the mature mRNA (34). The combinatorial exons, each 12–18 bp long, are among the smallest reported (34). However, the cardiac troponin T (TnT) gene has an exon of only six nucleotides in length (38). A previously reported B. mori chitinase cDNA (7) incorporates the 112-bp direct repeats but is devoid of the 9 bp, whereas the other reported cDNA from the same insect (8) lacks the upstream 121-bp direct repeat. All of these results together support the possibility of splicing of the 9 bp separately and irrespective of the other sequences of the primary transcript. Furthermore, exclusion of this insertion from the primary transcript yields a mature mRNA that encodes a protein 22 amino acids longer than that from a mature transcript including the insertion due to generation of a premature stop codon (Figs. 3 and 6). Exclusion of the triplet (GTG) at the exon 1 and intron 1 boundary and retention of the 9 bp at the 3′ region appeared more commonly in the mature transcripts from the late stages of the fifth instar larvae. However, generally, the alternative splicing phenomenon in the B. mori chitinase gene is not dependent on the fifth instar developmental stage (Table II).

To assess the changes in the levels of chitinase mRNAs in the fifth instar larvae epidermal tissues, total RNAs from the re-
spective tissues were analyzed by Northern blotting. Chitinase mRNA was undetected by Northern blotting on the day when the fifth instar larvae started spinning behavior. However, it was present at a very low level 1 day later, and its level increased sharply on the second day and gradually onward (Fig. 8A). The mature mRNAs expressing chitinase were also detected by RT-PCR (Fig. 8B). These data demonstrated the chitinase mRNA was transcribed even at the SP-0 stage, and it was detectable by RT-PCR when using only half of the amount of total RNA that was used in Northern blotting. This result strongly supports the concept of a gradual expression of the chitinase mRNA in B. mori from the day when the fifth instar larvae initiated spinning behavior and also supports the development-specific expression of chitinase, which was also detected in M. sexta larvae (6).

Two mechanisms were proposed for the generation of multiple functional chitinases from a single gene in B. mori. The chitinases may be processed from a larger one by limited proteolysis from the C-terminal side (16). Another mechanism is that the regulation of the enzymes expression at the gene level through alternative splicing of pre-mRNA. We present here the data that prove the alternative splicing mechanism. The role of this splicing mechanism in generating multiple functional chitinases in the silkworm was further demonstrated by expressing the alternatively spliced cDNA clones in E. coli. Chitinase activity of the four constructs was detected (Fig. 9B). This result reveals the role of alternative splicing of the primary transcript of the B. mori gene for chitinase in generation of multiple functional chitinase isoforms from a single gene, which is also consistent with three active chitinase isozymes expressed in vivo (16, 17). The post-translational modifications of M. sexta chitinase (39) and the glycosylation of the molting fluid chitinase (40) also give more support to this finding.

In conclusion, this study reports the involvement of alternative splicing of the primary transcript in generating multiple functional chitinase isoforms in the silkworm. The presence of this splicing mechanism in the B. mori gene for the chitinase also contributes to the interpretation of the variations reported in studies of the insect’s cDNAs. All of the mRNA products are from a single gene and functionally active.

Acknowledgments—We are very grateful to Drs. Karl J. Kramer, Grant Marketing and Production Research Center, Agricultural Research Service, United States Department of Agriculture, Manhattan, KS, and to Michael R. Kanost and Subbaratnam Muthukrishnan, Department of Biochemistry, Kansas State University, for peer reviewing the manuscript and critical comments. We acknowledge the staff at the DNA core facility of the Center for Gene Research, Yamaguchi University, for providing the competent cells.

REFERENCES

1. Koga, D., Mitsuomi, M., Kono, M., and Matsumiya, M. (1999) Chitin and Chitinases, Birkhauser Verlag, Basel/Switzerland, pp. 111–123

2. Kimura, S., (1973) J. Insect Physiol. 19, 115–123

3. Koga, D., Funakoshi, T., Mizuki, K., Utsumi, T., and Ide, A. (1989) Insect Biochem. 19, 123–128

4. Kramer, K. J., Muthukrishnan, S., Johnson, L., and White, F. (1996) in Advances in Insect Control: The Role of Transgenic Plants (Carozzi, N., and Koziel, M., eds) pp. 185–193, Taylor and Francis Publishers, Washington, D.C.

5. Overdijk, B., and Van Steijn, G. J. (1994) Glycobiology 4, 797–803

6. Kramer, K. J., Corpuz, L. M., Choi, H., and Muthukrishnan, S. (1993) Insect Biochem. Mol. Biol. 23, 681–701

7. Kim, M. G., Shin, S. W., Bae, K. S., Kim, S. C., and Park, H.-Y. (1998) Insect Biochem. Mol. Biol. 28, 163–171

8. Miki, K., Sugasaki, T., Shimada, T., Kobayashi, M., and Gustafsson, J.-A. (2000) J. Biol. Chem. 275, 37725–37732

9. Abdel-Banat, B. M. A., and Koga, D. (2001) Insect Biochem. Mol. Biol. 31, 497–508

10. Bogo, M. R., Rota, C. A., Pinto, H., Jr., Ocampo, M., Correa, C. T., Varinstein, M. H., and Schrank, A. (1998) Curr. Microbiol. 37, 221–225

11. Fuhrman, J. A. (1995) Parasitol. Today 11, 259–261

12. Watanabe, T., Kohori, K., Kiyotaka, M., Fuji, T., Sakai, H., Uchida, M., and Tanaka, H. (1993) J. Biol. Chem. 268, 15857–15872

13. Tellam, R. L. (1996) Parasitol. Today 12, 291–292

14. Rogers, S. G., Horsch, R. B., and Fraley, R. T. (1986) Methods Enzymol. 118, 627–640

15. Kramer, K. J., and Muthukrishnan, S. (1997) Insect Biochem. Mol. Biol. 27, 875–900

16. Koga, D., Sasaki, Y., Uchiumi, Y., Hirai, N., Arakane, Y., and Nagamatsu, Y. (1997) Insect Biochem. Mol. Biol. 27, 757–767

17. Abdel-Banat, B. M. A., Kameyama, Y., Yoshioka, T., and Koga, D. (1999) Insect Biochem. Mol. Biol. 29, 537–547

18. Choi, H. K., Choi, K. H., Kramer, K. J., and Muthukrishnan, S. (1997) Insect Biochem. Mol. Biol. 27, 37–47

19. Jiang, H., Wang, Y., and Kanost, M. R. (1994) J. Biol. Chem. 269, 55–58

20. Dirksen, W. P., Hampson, R. K., Sun, Q., and Rottman, F. M. (1994) J. Biol. Chem. 269, 6431–6436

21. Jiang, H., Wang, Y., Huang, Y., Mulnix, A. B., Kandel, J., Cole, K., and Kanost, M. R. (1996) J. Biol. Chem. 271, 28017–28023

22. Stern, D. A., and Bergert, S. M. (1993) Mol. Cell. Biol. 13, 2677–2687

23. Green, M. R. (1991) Annu. Rev. Cell Biol. 7, 559–599

24. Chang, D. D., and Sharp, P. A. (1989) Cell 59, 789–795

25. Hamm, J., and Mattaj, I. W. (1990) Cell 63, 109–118

26. Legrain, P., and Rooshash, M. (1989) Cell 57, 573–583

27. Cooke, N. E., Ray, J., Emery, G. J., and Liebhaber, S. A. (1988) J. Biol. Chem. 263, 9001–9006

28. Craibtree, G. R., and Kent, J. A. (1982) Cell 31, 159–166

29. Hampson, R. K., and Rottman, F. M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2673–2677

30. Weil, D., Brosset, S., and Dautry, F. (1990) Mol. Cell. Biol. 10, 5865–5876

31. Michaud, S., and Reed, R. (1995) Genes Dev. 7, 1008–1020

32. Koga, D., Hirata, T., Sueshige, N., Tanaka, S., and Ide, A. (1992) Biosci. Biotechnol. Biochem. 56, 280–285

33. Kramer, K. J., Dziadik-Turner, C., and Koga, D. (1985) in Comprehensive Insect Physiology, Biochemistry and Pharmacology (Kerkut, G. A., and Gilbert, L. I., eds) Vol. 3, pp. 171–163, Pergamon Press, New York

34. Breitbart, R. E., Andreatis, A., and Nadal-Ginard, B. (1987) Ann. Rev. Biochem. 56, 467–495

35. Ranson, H., Collins, F., and Hemingway, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14284–14289

36. Pongjaroenkit, S., Jriraroenrat, K., Boonchaoy, C., Chanama, U., Leetachewa, S., Prapanthadara, L., and Ketterman, A. J. (2001) Insect Biochem. Mol. Biol. 31, 75–85

37. Garel, A., Deleage, G., and Prudhomme, J. C. (1997) Insect Biochem. Mol. Biol. 27, 469–477

38. Cooper, T. A., and Ordahl, C. P. (1985) J. Biol. Chem. 260, 11140–11148

39. Gopalakrishnan, B., Muthukrishnan, S., and Kramer, J. K. (1995) Insect Biochem. Mol. Biol. 25, 255–265

40. Koga, D., Jilka, J., and Kramer, J. K. (1983b) Insect Biochem. 13, 295–305
Alternative Splicing of the Primary Transcript Generates Heterogeneity within the Products of the Gene for *Bombyx mori* Chitinase

Babiker M. A. Abdel-Banat and Daizo Koga

*J. Biol. Chem.* 2002, 277:30524-30534.
doi: 10.1074/jbc.M112422200 originally published online June 3, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M112422200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 12 of which can be accessed free at http://www.jbc.org/content/277/34/30524.full.html#ref-list-1