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Isolation and Characterization of a Genomic Clone for the Gene of an Insect Molting Enzyme, Chitinase

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Genomic clones for a chitinolytic enzyme were isolated from a library of *Sau* 3A digested DNA from the tobacco hornworm, *Manduca sexta*, using a previously isolated chitinase cDNA clone as a probe [Kramer *et al.*, *Insect Biochem. Molec. Biol.* 23, 691–701 (1993)]. Restriction enzyme mapping and Southern blot analysis of four genomic clones suggested that these are overlapping clones. Sequence analysis of the genomic clones and Southern blot analysis of total genomic DNA also suggest that the *M. sexta* genome has only one chitinase gene detectable by the cDNA probe. This gene is organized into at least 11 exons in a region spanning >11 kb. The sequenced *M. sexta* chitinase gene has a series of exons corresponding to identifiable structural/functional regions of the protein. Similarities in structure and organization between the *M. sexta* chitinase gene and chitinase genes from other sources are described. Published by Elsevier Science Ltd

Chitinase *Manduca sexta* Tobacco hornworm Genomic clone DNA sequence Molting enzyme Gene structure Exon Intron Chitinolytic enzyme Chitinase

INTRODUCTION

Chitin is one of the most abundant polysaccharides in nature and has been found in the exoskeletons and gut linings of insects, cell walls of fungi, and shells of crustaceans. It is a linear polymer of β (1→4) linked *N*-acetylglucosamine (GlcNAc) residues. Chitinolytic enzymes that catalyze the hydrolysis of chitin have been found in chitin-containing organisms as well as in microorganisms, plants, and animals that do not have chitin. The enzymes from different sources have different biological functions, such as molting of the exoskeleton in insects and crustaceans, cell growth and division in fungi, utilization of chitin for nutrition in bacteria, and defense against pest and pathogen attacks in plants (Flach *et al.*, 1992).

The enzymatic degradation of chitin is a complex process. In the tobacco hornworm, *Manduca sexta*, a binary mixture of chitinase and β-*N*-acetylglucosaminidase hydrolyzes chitin (Fukamizo and Kramer, 1985a, b). The two enzymes exhibit a synergism such that the rate of hydrolysis of chitin by the mixture of two enzymes is as much as six times faster than the sum of the individual enzyme's rates. The endosplitting chitinase initiates the hydrolysis of chitin and produces oligosaccharides. The intermediate oligosaccharides are converted to GlcNAc by the exosplitting β-*N*-acetylglucosaminidase.

A cDNA clone encoding a chitinolytic enzyme from the tobacco hornworm, *Manduca sexta*, was isolated and characterized in our laboratory. Using this cDNA clone as a probe, the tissue specificity and hormonal regulation of expression of the chitinase gene during development were studied (Kramer *et al.*, 1993). In order to extend our understanding of the structure of chitinase genes in insects and to help determine how they are regulated, genomic clones containing chitinase genes were isolated from *M. sexta* and characterized in this research. The organization of the gene and encoded insect chitinase was compared to that of chitinolytic enzymes from other species.

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MATERIALS AND METHODS

Insect rearing and DNA isolation

Manduca sexta eggs were obtained from the Biosciences Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota. Larvae were raised on an artificial diet at 27°C according to Bell and Joachim (1976). Genomic DNA was isolated from day 4 fifth instar larvae as described previously (Kramer *et al.*, 1993).

Genomic library construction

Random fragmentation of high molecular weight *M. sexta* genomic DNA was performed by *Sau* 3A partial digestion. DNA fragments with a size range of 9–23 kb were recovered by gel electrophoresis and were used for ligation with λ-EMBL3 arms and packaged using Gigapack II packaging extracts (Stratagene, La Jolla, CA) following standard procedures (Sambrook *et al.*, 1989).

Library screening

Two different unamplified genomic libraries were screened with the ^{32}P-labeled 1.8 kb *Eco RI* fragment from the *M. sexta* chitinase cDNA clone 201 containing the protein coding sequences (Kramer *et al.*, 1993). From 3×10^5 plaques from the first genomic library, three positive plaques were isolated. From a similar number of plaques obtained from a second library independently prepared by Dr Michael Kanost (Kansas State University), an additional clone (G1) was obtained. All four of the clones were plaque-purified and were named G203, G207, G216, and G1.

Probes for Southern blotting

The first probe was a short DNA fragment corresponding to the 200 nucleotides at the 5′-end of chitinase cDNA clone 201. It was obtained by amplification using a polymerase chain reaction (PCR) of clone 201 DNA with T7 universal primer and another primer complementary to a 17 nucleotide-long sequence that was located about 200 nucleotides downstream from the 5′-end of clone 201 (Kramer *et al.*, 1993). A second probe was the insert of clone G14, a subclone of chitinase genomic clone G1 (see below) from the middle of the chitinase gene, and was prepared from clone G14 plasmid DNA by *Sal* I digestion. Other probes were the 1.8-kb *Eco RI* fragment of cDNA clone 201, the 6.5-kb *Sal* I fragment of G1, and the 3-kb *Sal* I fragment of another genomic clone, G207 (see Fig. 1 for location of G1 probes). The probe fragments were labeled with [α-^{32}P]-dCTP by the random prime labeling method (Feinberg and Vogelstein, 1983).

Southern blotting

Genomic clones G1, G203, G207, and G216 were digested by *Sal* I restriction enzyme. Digested DNAs (2 μg) were subjected to 0.8% agarose gel electrophoresis and blotted on to a nitrocellulose membrane (Southern, 1975). The membrane was prehybridized, hybridized in 6×SSC (SSC equals 0.15 M NaCl and 0.03 M sodium citrate pH 8.0), washed at high stringency (0.1×SSC at 65°C), and was exposed to an X-ray film. After stripping the first DNA probe, the membrane was used sequentially for hybridization with other probes.

Genomic clone sequencing

The insert DNAs in the genomic clones were recovered by *Sal* I digestion of λ-DNAs and subcloning in pBluescript KS$^+$ vector (Stratagene, La Jolla, CA). Sequencing of subclones was performed by two approaches. For target DNA shorter than 2 kb, initial sequencing was done using T3 and T7 primers. If a suitable restriction enzyme site was present within the sequence obtained by using T3 or T7 primers, sequences up to this site in the target DNA were removed by appropriate restriction enzyme digestion and religation of the plasmid DNA. The target DNA was sequenced again using T3 or T7 primers. If no suitable restriction sites were found within the newly determined sequence, a custom oligonucleotide primer was designed from the data obtained in the previous round of sequencing. Sequencing was completed in a step-wise fashion by repeating the above procedure.

When target DNA was over 2 kb, a nested set of deletion clones was generated by digestion of the pBluescript plasmid containing the target DNA fragment with exonuclease III followed by ligation and transformation (Rogers and Weiss, 1980). Deletion clones with progressively shorter insert fragments were sequenced. Gaps in the sequences were filled by sequencing the original insert DNA fragments with custom synthesized primers.

RESULTS

Southern blot analysis of genomic clones

λ-DNAs from clones G1, G203, G207, and G216 were subjected to digestion with the restriction enzyme *Sal* I. Four insert fragments of sizes 7, 6.5, 2, and 1 kb were obtained from clone G1. Clone G203 released fragments of sizes 12, 4.8, and 1 kb besides the vector arms. *Sal* I digestion of clone G207 produced two insert fragments of sizes 10 and 3 kb. Clone 216 has three insert fragments of 12, 1.2, and 1 kb. Only the 1.0 kb fragment is common to clones G1, G203, and G216, but this fragment is missing in G207.

Southern blot analysis of the *Sal* I digest of DNA from clone G1 revealed that all insert fragments except the 2-kb fragment hybridized with the 1.8-kb *Eco RI* fragment of chitinase cDNA clone 201 (data not shown). To establish the relationship of various fragments of G1 with those of other chitinase genomic clones and to identify chitinase coding sequences, Southern blot analyses of *Sal* I digests of the λ-DNAs were carried out with different

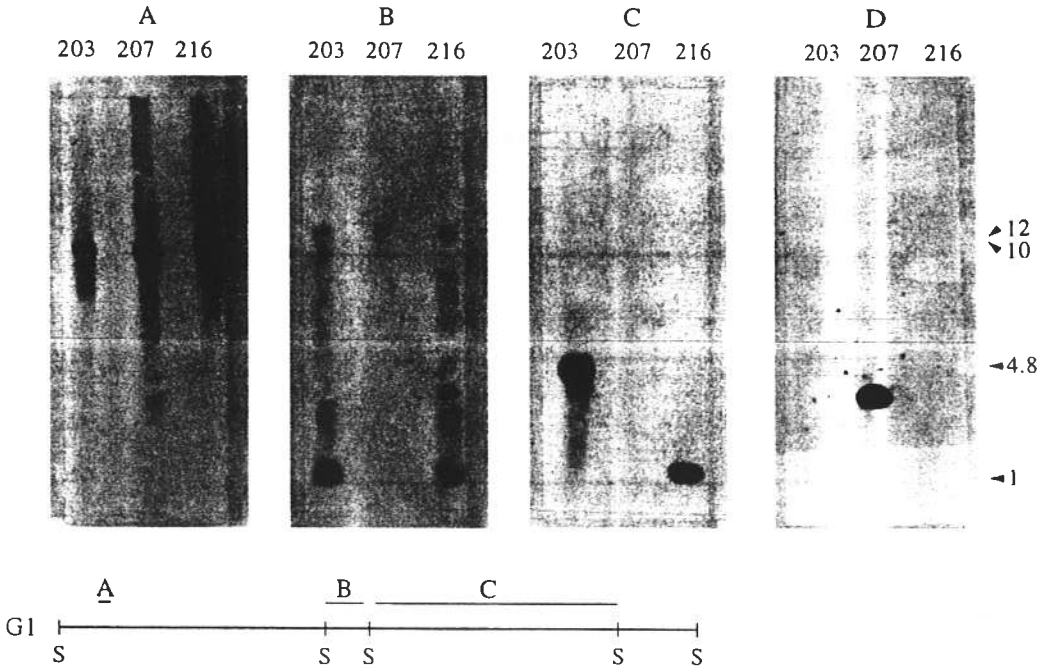

FIGURE 1. Southern blot analysis of *M. sexta* genomic clones G203, G207, and G216 digested by *Sal* I enzyme. Sizes in kilobases (kb) are indicated on the right. The diagram below the Southern blots shows the location of the three hybridization probes in genomic clone G1. The fourth probe was from clone G207. The probes used were: panel A, a 200-bp-long PCR fragment from the 5′-end of cDNA clone 201; panel B, the 1-kb *Sal* I fragment of genomic clone G1; panel C, the 6.5-kb *Sal* I fragment of genomic clone G1; and panel D, the 3-kb *Sal* I fragment of genomic clone G207. The autoradiogram was exposed for 12 h. The minor band of size >12 kb in lane 207 of panel A is due to incomplete digestion.

probes. The 1-kb *Sal* I fragment of clone G1 hybridized to the 1-kb fragments of clone G203 and G216, indicating that these fragments were related, if not identical (Fig. 1, panel B). When a PCR product, corresponding to the 5′-end of the chitinase cDNA clone 201, was used as the probe, the 12-kb *Sal* I fragment of G203, the 10-kb *Sal* I fragment of G207, and the 12-kb *Sal* I fragment of G216 hybridized to the 5′-end probe (Fig. 1, panel A, fragment A in the bottom diagram), indicating the presence of 5′-terminal sequences of the chitinase cDNA in all of the genomic clones. The 7-kb *Sal* I fragment of G1 also hybridized to this probe (data not shown). Clone G207 did not hybridize with the 6.5-kb G1 probe, whereas clones 203 and 216 showed hybridization to their 4.8-kb and 1.2-kb fragments, respectively (Panel C). The 3-kb *Sal* I fragment clone from G207 did not hybridize with any other fragments except itself (Panel D). From these analyses and restriction enzyme digestion data, the *Sal* I restriction maps of clones G1, G203, G207, and G216 and their relationship to one another are inferred to be as outlined in Fig. 2. These data indicate that clones G1, G203, G216, and probably G207 are overlapping and contain the same chitinase gene. Because genomic clone G1 appeared to contain the 5′-sequence of the chitinase cDNA and extended the farthest in the 3′-direction, an extensive sequence analysis of this clone was carried out.

DNA sequence of chitinase gene in genomic clone G1

The 7, 6.5, and 1-kb *Sal* I fragments of clone G1 that hybridized to the chitinase cDNA probe were subcloned in the pBluescript KS+ vector and denoted as G11, G12, and G14, respectively. G11 has one internal *Bam HI* restriction site in the insert, which cut the 7-kb *Sal* I fragment into 3.7-kb and 3.3-kb fragments. These two fragments were used to obtain subclones B1 and B2. The 6.5-kb fragment in G12 contains an internal *Bam HI* and two *Xho* I sites. These sites were utilized to obtain additional subclones. The complete sequences of clones G11, G12, and G14 were determined and combined to obtain the sequence of the chitinase gene encoded in genomic clone G1 (Fig. 3). To confirm the correctness of the orientation of the fragments in the combined sequence, additional subclones flanking the joints were sequenced using synthetic primers. The sequence has been submitted to GenBank (Accession no. L49234).

Structure of a chitinase gene from M. sexta

A comparison of the DNA sequence of clone G1 with that of cDNA clone 201 confirmed the ordering of the *Sal* I fragments in clone G1. The sequenced data also revealed that the chitinase gene in G1 consists of 10 exons and nine introns covering a stretch of approximately 11 kb. Exon 1 has the start codon, ATG (positions 2573–2575), and encodes the entire signal pep-

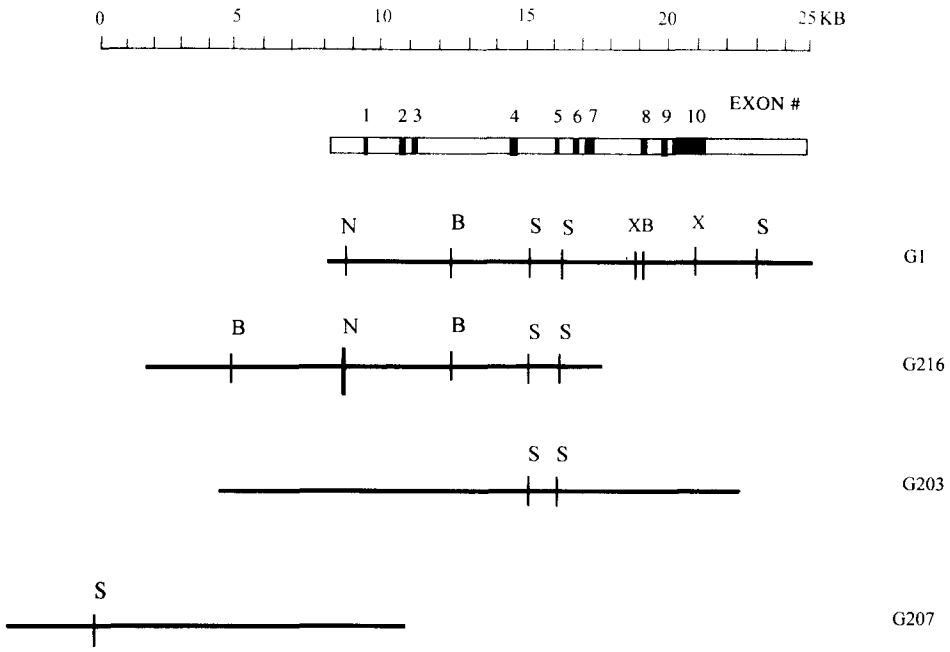

FIGURE 2. *Sal* I restriction maps of genomic clones G1, G203, G207, and G216 (S, *Sal* I; B, *Bam HI*; X, *Xho* I; and N, *NsiI*). A schematic diagram of the *M. sexta* chitinase gene in clone G1 is shown below the size scale. Exons (1–10) are marked by dark boxes. The alignment of clone G207 is arbitrary except that it contains sequences that are detectable by a 200-bp PCR fragment corresponding to the 5′-end of cDNA clone 201.

tide of 19 amino acids. Exon 2 begins with the mature N-terminal sequence of *M. sexta* chitinase, DSRARIV (Gopalakrishnan *et al.*, 1995). Exon 3 includes a sequence that encodes a conserved motif, KFMVAVGGWAEGS, found in many chitinases, and exon 4 has the sequence of another conserved motif, YDFDGLDLDWEYP, presumed to be part of the catalytic site (Kuranda and Robbins, 1991; Watanabe *et al.*, 1993). Exon 10 has a stop codon (positions 12645–12647) and encodes a long untranslated region of 750 nucleotides. The junction sequences where the exons and introns meet have features characteristic of eukaryotic pre-mRNA splice junctions. The sizes of the introns are in the range 0.6–1.5 kb, except for intron 3, which is 3.35 kb long. Table 1 shows the sizes of the exons, protein sequences encoded, and their special features.

When the intron sequences are removed from the genomic clone, the sequence of the chitinase gene in clone G1 matches precisely that of the chitinase cDNA clone 201 from nucleotide position 21 of the cDNA clone to its 3′-end at position 2652 (Kramer *et al.*, 1993). The region of sequence identity includes 750 nucleotides of the 3′-untranslated region, indicating that clone G1 corresponds to the cDNA clone 201. However, the first 20 base pairs of the cDNA insert of clone 201 (excluding the *EcoRI* linker) are not found in clone G1, even though it extends more than 1200 nucleotides upstream of the ATG start codon.

The restriction maps of clones G1 and G216 indicate that they are overlapping clones (Fig. 2). This was confirmed by direct DNA sequencing of subclones of the 12-kb *Sal* I fragment of clone G216 using several synthetic primers. These sequences are identical to the corresponding regions of G1, confirming that clones G1 and G216 are overlapping and contain the same chitinase gene. We sequenced additional fragments contained in genomic clone 216, which extends 5.1 kb further upstream of the G1 clone. So far, we have sequenced approximately 2500 nucleotides upstream of the ATG start codon, but have failed to detect sequences corresponding to the 5′-terminal 20 nucleotide sequence of cDNA clone 201.

Two oligonucleotides, whose sequences are complementary to the sequence of the first 30 nucleotides of the 5′-end of cDNA clone 201, were synthesized and used as primers for sequencing plasmid DNAs from genomic subclones presumed to contain the 5′-upstream region. However, the custom primers failed to yield primer extension products with any of the fragments from genomic clones G1 or G216, suggesting the absence of primer binding sites in them.

Comparison of the intron sequences of genomic clones G1 and G207

Based on restriction map and some limited sequencing data, we could conclude that clones G1, G203, and G216 are overlapping clones. However, available data could not eliminate the possibility that clone G207 contains a different chitinase gene. To check the possibility that the genomic clones G1 and G207 contain different chitinase genes, limited sequencing of approximately 200 nucleotides of the chitinase genes contained in them was carried out using a primer complementary to the sequence of exon 2 of clone G1. The region sequenced corresponded to a region in the first intron. A comparison of the two sequences revealed that the sequences are identical, except for a couple of ambiguities in the sequencing reac-

Nucleic Acid Sequence of a chitinase gene from *M. sexta*

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agaaaccgttaaagtaagtcgatctgctacttaaaaactctttaattacgaaacttaacgccattaccctatagatgaagccccttcccctaaaacggat 15000
atctgaaactaccaaattaaattccagctatttgccgattcaatcctatatttatattcaaaggtgcttaaatactgtacctactctaaataaacgttga 15100
taacccattcaaattgaaattactacggacccacggtcgggcaaaagacctttttagtcgcataacgtaaatagcacgctactttgaactttgtttttta 15200
tctaccatgtatctcaggttatttttaaacgtgctttatatattttcatggtgaggcaggatgtttaacctttcaccaattatatgtcggcaattatacg 15300
tcgatgcacctatttatgttatgacgtttaagacaattttgttatgtgtgagtaaattaaaagagtggtgttacaaaatgttaggttaaaatgctaattg 15400
gaatggcttcggcgtgttcggttagaagtctcggagtcattgtaaaatgctgatagagagcaccatgggggttttagtttttaaaaatccgacatactc 15500
cctctaccttgtatatgcacaaaggaaccaagaggattttctctatgaaaaaaaatcggtcgacctcgcagccgggccgtaccagcttttcgttcccctt 15600
tagtcgagggttacctgtgtgaaattgttacc 15632
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FIGURE 3. Continued.

TABLE 1. Special features of exons from *M. sexta* chitinase genomic clone G1

Exon	size (in bp)	Size (in aa)	Protein sequence encoded*	Special features
1	62	19	MRATLATLAVLALATAVQS	Start codon (ATG) and signal peptide
2	170	56	DSRARIVCYFSNWAVYRPGVGRYGIEDIPVEKCTHIIYSFIGVYEGNSEVLIIDPE	Mature N-terminus
3	166	56	LDVDKNGFR**NFT**SLRSSHPSV**KFMVAVGGWAEGS**SKYSHMVAQKSTRMSFIRSVVS	Possible N-glycosylation site and conserved region I
4	233	74	FLKK**YDFDGLDLDWEYP**GAADRGGSFSDKDKFLYLVQELRRAFIRVFKGWELTAAVPLANFRLMEGYHVPELCQ	Conserved region II
5	124	41	ELDAIHVM**SYD**LRGNWAGFADVHSPLYKRPHDQWAYEKLNV	Possible phosphorylation site
6	192	64	NDGLHLWEEKGCPSNKLVVGIPFYGRSFTLSAGNNNYGLGTFINKEAGGGDPAPYT**NAT**GFWAY	Possible N-glycosylation site
7	302	101	YEICTEVDKDDSGWTKKWDFQGKCPYAYKGTQWVGYEDPR**SVE**IKMNWIKQKGLYGAMTWAIDMDDFQGLC-GEKNPLIKILHKHMSSYTVPPPHTE**NTTPT**	Possible phosphorylation site and possible N-glycosylation site
8	231	77	**PE**WARPP**STPS**DPSEGDPIP**TTTT**AKPA**STTKTT**VK**TTTTTT**AKPPQ**S**VIDEENDINVRPEPKPEPQPEPEVEVP**PT**	Threonine/serine-rich region
9	75	25	ENEVDGSE**ICN**SDQDYIPDKKH**CDK**	Cysteine-rich region
10	873	41	YWR**C**VNGEAMQFS**C**QHGTVFNVELNV**C**DWPS**NAT**RRE**C**QQP	Cysteine-rich region, possible N-glycosylation site, termination codon (TAA), and untranslated region

*Special features are shown in bold lettering.

tion. These data suggest that the two chitinase genes in clones G1 and G207 are the same.

Southern blot analysis of M. sexta *genomic DNA*

Southern blot analysis of the *Bam* HI digested genomic DNA from *M. sexta* was done using the 1.8-kb *Eco RI* fragment of cDNA clone 201 and two *Bam HI* subfragments of this DNA with sizes of 1.3 (5′-fragment) and 0.5 kb (3′-fragment) as hybridization probes. From the sequence data and restriction enzyme analysis of overlapping genomic clones G1 and G216, three *Bam HI* sites were found to be present in this chitinase gene (one 5′ to exon 1, one in intron 3, and one in exon 8; see Fig. 2 for location of the *Bam HI* sites). If the *M. sexta* genome contains a single chitinase gene represented by clone G1 (and G216), digestion with *Bam HI* should produce the 7.9 and 6.8-kb fragments and one other 3′-fragment of unknown size detectable by the 1.8-kb probe. This probe detected the two bands of sizes 7.9 kb, 6.8 kb, and two others with sizes >15 kb, instead of one as predicted (Fig. 4, lane 1). The 1.3-kb 5′-*Bam HI* probe detected only the 7.9 and 6.8-kb fragments, but not the two >15-kb bands as expected (Fig. 4, lane 2). The 0.5-kb 3′-fragment probe detected both bands of sizes >15 kb, which also were detected by the full length probe (Fig. 4, lane 3).

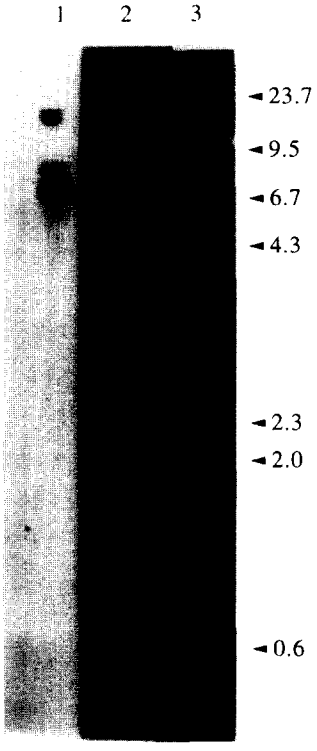

FIGURE 4. Southern blot analysis of *Bam HI* digested *M. sexta* genomic DNA using ^{32}P-labeled 1.8-kb *EcoRI* fragment (lane 1), 5′-*Bam HI* fragment (1.3 kb) (lane 2), and 3′-*Bam HI* fragment (0.5 kb) (lane 3) from clone 201 as probes. The blot was probed sequentially with the indicated fragments. Detection of the 6.8 and 7.9-kb bands in lane 3 is due to incomplete removal of the probe from the previous round of hybridization with the 1.3-kb probe.

DISCUSSION

Screening of the *M. sexta* genomic libraries with a chitinase cDNA probe resulted in the isolation of four clones containing chitinase sequences. These appear to be overlapping clones with different 5′- and 3′-end points, based on the identity of restriction site distributions, Southern blot analyses, and limited DNA sequence comparisons including a portion of the first introns of clones G1 and G207.

Southern blot analysis of *M. sexta* genomic DNA with the full length cDNA and its 5′ and 3′ *Bam HI* fragments led to ambiguous results. The 5′-probe yielded two fragments of sizes 7.9 and 6.8 kb as expected from a single chitinase gene contained in clones G1 and G216. On the other hand, the *Bam HI* probe corresponding to the 3′-end of the chitinase cDNA detected two fragments instead of the expected single fragment in *Bam HI* digests of genomic DNA, suggesting the presence of a second gene. However, we did not find evidence for a second gene from sequence analysis of the cDNA clones. Furthermore, the limited sequencing carried out with the genomic clones has failed to reveal the presence of another chitinase gene. One possible explanation for the Southern blot ambiguity is that the detection of two bands by the 3′-probe is due to allelic variation in the location of a *Bam HI* site that lies 3′ to the chitinase gene. The allelic variation is most likely due to the fact that the DNA used in the Southern blotting experiments was obtained from a mixture of several larvae. However, the possibility that there is a second chitinase gene related to chitinase cDNA clone 201 cannot be eliminated by the available data.

The DNA sequence alignment of genomic clone G1 with cDNA clone 201, which is a full-length chitinolytic enzyme cDNA from *M. sexta*, reveals the correspondence of the two clones, except that genomic clone G1 is missing the first 20 nucleotides of the 5′-end of the cDNA clone 201 insert. Genomic clone G1 has all of the chitinase coding region and the 3′-untranslated region of clone 201. The absence of mismatches between these clones in both the coding and untranslated regions confirms that cDNA clone 201 and genomic clone G1 represent the same chitinase gene.

Even though we have sequenced more than 2.5 kb of the region 5′ to the ATG start codon, the sequence corresponding to the first 20 nucleotides of the chitinase cDNA clone 201 has not been identified. The sequence that precedes the first identified exon has the characteristics of the 3′-end of an intron. Furthermore, there are no identifiable "TATA"-box sequences just upstream of "exon 1". Thus, it appears that "exon 1" is preceded by a large intron and at least one other exon. Our attempts to identify the sequences corresponding to these 20 nucleotides in the genomic clones have been unsuccessful using extension of primers complementary to the cDNA 5′-end sequences with any of the genomic clone fragments as templates including clone G216 that extends

more than 5 kb in the 5′-direction relative to clone G1. Additional experiments such as probing genomic DNA or clones using an oligonucleotide corresponding to the missing region as the labeled probe might help to identify the transcription start site.

The chitinase gene in genomic clone G1 shows structural features common to eukaryotic genes. The intron–exon splice junctions have the expected conserved sequences. Each distinctive domain of the chitinase appears to be encoded in separate exons. Exon 1 starts 7 bp upstream of the ATG start codon and encodes the whole hydrophobic signal peptide of the enzyme. This indicates an unusually short 5′-untranslated region in chitinase RNA. However, the 5′-untranslated region might be longer than seven nucleotides if additional exons are identified. The second exon begins with the mature N-terminus of chitinase. The polypeptide regions, I and II, which are highly conserved in chitinases, are located in exons 3 and 4, respectively, and the threonine/serine-rich region is encoded in exon 8. The C-terminal cysteine-rich region and the entire 3′-untranslated region reside in exon 10. The C-terminal cysteine-rich region might be a chitin-binding domain based on the analogy with some chitinases and lectins that have an N-terminal cysteine-rich region believed to contribute to their strong affinity for chitin (Chrispeels and Raikhel, 1991; Kuranda and Robbins, 1991). The 85-kDa *M. sexta* chitinase can be resolved into isoforms differing in p*I*, but it is unclear whether this is due to phosphorylation or some other chemical difference (Wang et al., manuscript in preparation). Several chitinases are synthesized as zymogens and become more active upon proteolysis (Koga *et al.*, 1989, 1992), but *M. sexta* chitinase encoded in cDNA clone 201 appears not to have a zymogenic form (Kramer *et al.*, 1993; Gopalakrishnan *et al.*, 1995). There is a consensus polyadenylation signal at a position 22 nucleotides upstream of the end of exon 10.

Manduca sexta chitinase has a structural organization similar to that of *Saccharomyces cerevisiae* chitinase, which has four distinctive regions including a signal sequence, a region presumed to contain the catalytic domain, a serine/threonine-rich region, and a carboxyl-terminal region (Kuranda and Robbins, 1991). Exons 5, 7, and 8 have possible phosphorylation sites that may be involved in the regulation of chitinase activity. The carboxyl terminal portion of yeast chitinase is apparently responsible for a high binding affinity for chitin. On the other hand, some plant chitinases have an N-terminal chitin-binding domain rich in cysteines. They are encoded in small regions of DNA of 2–4 kb in length and apparently have none or only a few (one or two) introns. Even though plant chitinase genes are believed to have evolved from an ancestral gene and have multiple domains, they do not appear to possess multiple exons (Gaynor and Unkenholz, 1989; Shinshi *et al.*, 1990). They are also significantly smaller proteins, with sizes ranging from 20 to 36 kDa. The organization of other insect chitinase genes is not known at present. It will be interesting to determine whether insect chitinase genes as a class are more complex than their counterparts in plants.

Because the chitinase gene is regulated by ecdysteroid hormones (Kramer *et al.*, 1993), the 5′-sequence in front of exon 1 as well as the rest of the gene was examined for ecdysteroid-responsive elements that have the consensus sequence (the sequences of both strands were analyzed), RGG/TTCANTGAC/ACY (Cherbas *et al.*, 1991). Such a sequence was unapparent in clone G1. The hormonally responsive elements might be located upstream of the 5′-region sequenced and upstream of the first exon that is yet to be identified. Alternatively, the hormone-responsive elements in the *M. sexta* chitinase gene might not be related closely to the consensus sequence that contains several degenerate positions.

A search of the databases with the BLASTP program (Altschul *et al.*, 1990) for proteins with sequence similarity to *M. sexta* chitinase resulted in the identification of a venom gland chitinase from *Chelonus* sp. (a wasp, Krishnan *et al.*, 1994); a chitinase from *Brugia malayi* (a nematode, Fuhrman *et al.*, 1992); a fungal chitinase (Blaiseau and Lafay, 1992); and several glycoproteins, including one from *Drosophila melanogaster* (Kirkpatrick *et al.*, 1995) and eight from mammalian species (Malette *et al.*, 1995). Pair-wise comparisons (data not shown) show that wasp chitinase, nematode chi-

SOURCE	SCIENTIFIC NAME	REGION I	REGION II (ACTIVE SITE)
Insect	*Manduca sexta*	(97) K F M V A V G G W A E G S	(136) Y D F D G L D L D W E Y P
Wasp Venom	*Chelonus sp*	(96) K I M V A V G G W N A G S	(135) Y Q F D G F D I D W E Y P
Nematode	*Brugia malayi*	(99) K V L L S Y G G Y N F G S	(138) N N F D G F D L D W E Y P
Fungus	*Aphanocladium album*	(89) K V M L S I G G W T W S T	(127) W G F D G I D I D W E Y P
		* * *	* * * * * * * * *
Insect	*Drosophila melanogaster*	(107) K I L L S V G G D K D I E	(155) Y G F D G L D V A W Q F P
Human	*Homo sapiens*	(91) K T L L S V G G W N F G S	(130) H G F D G L D L A W L Y P
Mouse	*Mus musculus*	(107) K T L L A I G G W K F G P	(131) Y N F D G L N L D W Q Y P
Hamster	*Mesocricetus auratus*	(70) K T L L S V G G W N F G T	(109) H G F D G L D L F F L Y P
Bovine	*Bos taurus*	(88) K T L L S I G G W N F G T	(128) H G F D G L D L F F L Y P
		* * * *	* * * *

FIGURE 5. Alignment of conserved regions I and II of *M. sexta*, *Chelonus* sp. (Krishnan *et al.*, 1994), *Brugia malayi* (Fuhrman *et al.*, 1992), and *A. album* (Blaiseau and Lafay, 1992) chitinases and glycoproteins from *Drosophila melanogaster* (DS 47, Kirkpatrick *et al.*, 1995), human cartilage (HC gp-39, Hakala *et al.*, 1993), murine macrophage (YM-1, Genbank accession #S27879), hamster (Genbank accession #V15048), and bovine sources (Genbank #D16639). * indicates residues conserved in each of the two groups. Numbers indicate positions in the amino acid sequence.

tinase, fungal chitinase, and the *Drosophila* glycoprotein had 37, 27, 26, and 20% identities with the amino acid sequence of *M. sexta* chitinase, respectively. A lower level of sequence similarity was observed with yeast, plant (classes III and V), and bacterial chitinases. The first two cysteine residues in the N-terminal sequences of the hornworm (positions 27 and 52), wasp, and nematode chitinases and the last six cysteines in the C-terminal sequences of the hornworm (positions 500, 513, 519, 529, 542, and 553) and nematode chitinases are conserved (Fuhrman, 1995). The similarities of conserved regions I and II (KFMVAVGGWAEGS and YDFDGLDLDWEYP) are also striking (Fig. 5). One K, three Gs, and one S in conserved region I are identical in the three chitinases. Conserved region II has a higher level of sequence conservation with nine identical residues (F, D, G, D, D, W, E, Y, P). In chitinases from *M. sexta*, nematode, and wasp, conserved region II has all identical residues, except for one Y, one D, and two L residues. The *Drosophila* and other glycoproteins, DS-47, HC-gp39 (Hakala *et al.*, 1993), and YM-1 (Genbank accession #S27879; Chang, personal communication), lack either the glutamic acid or aspartic acid within region II, which are residues proposed to be present at the active sites of chitinases (Watanabe *et al.*, 1993). It is noteworthy that this latter group of glycoproteins exhibits no chitinolytic activity and might have a glycan-recognition function that helps to protect cells from microbial pathogens (Kirkpatrick *et al.*, 1995; Malette *et al.*, 1995). The glycoproteins do have a degree of sequence conservation in region I, which is similar to that of the chitinases. Even though the *M. sexta* sequence exhibits a relatively low degree of similarity overall with two classes of chitinases from plants (classes III and V) and with chitinases from yeasts and fungi, conserved regions I and II are present in all of these chitinases. One glutamic acid and two aspartic acids in conserved region II are invariant in most of these enzymes. The *M. sexta* enzyme also shares significant sequence similarity with the N-terminal sequence and an internal region of a human chitotriosidase (Boot *et al.*, 1995; Renkema *et al.*, 1995).

The sequence of the threonine/serine-rich region in *M. sexta* is not highly conserved when compared to corresponding regions of wasp venom and nematode chitinases (Fuhrman *et al.*, 1992; Krishnan *et al.*, 1994). Nevertheless, all three of these chitinases are rich in serine and threonine in this region, some of which might represent O-glycosylation sites. It is interesting that the glycoproteins with sequence similarities to chitinases listed in Fig. 5 also have serine- and threonine-rich repeats in their carboxyl-terminal regions, which are heavily glycosylated (Malette *et al.*, 1995). These molecules are believed to interact with specific oligosaccharide ligands and, thus, be involved in promoting or inhibiting adhesion between different cell types.

Chitinases have received substantial attention because some of these enzymes have exhibited insecticidal and fungicidal activities. For example, *M. sexta* chitinase cDNA with a CaMV 35S promoter recently has been introduced into tobacco plants (Ding, 1995). The transgenic plants synthesize active chitinase constitutively and have enhanced resistance toward some insect species. A genetically engineered baculovirus expressing *M. sexta* chitinase also has been shown to possess enhanced insecticidal activity (Gopalakrishnan *et al.*, 1995). A fuller understanding of the structure and regulation of insect and other types of chitinases and their genes should facilitate the manipulation of these enzymes for the purpose of controlling insect and microbial pests.

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