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Cloning, Expression, and Hormonal Regulation of an Insect β-*N*-Acetylglucosaminidase Gene

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Chitinolytic enzymes such as β-*N*-acetylglucosaminidases are major hydrolases involved in insect molting. By screening a *Manduca sexta* (tobacco hornworm) cDNA library with an antibody against β-*N*-acetylglucosaminidase from molting fluid of *M. sexta* pharate pupae, several putative cDNA clones for this enzyme were isolated. The longest of the cDNA clones has an insert of approximately 3 kb, and the complete nucleotide sequence was determined. Because this clone is missing the initiation codon and nucleotides corresponding to the leader peptide, the mRNA 5′-end sequence was determined by PCR (polymerase chain reaction) amplification and cycle sequencing. The sequence of the encoded protein from positions 23 to 35 is identical to the NH_2-terminal sequence of one of the β-*N*-acetylglucosaminidases isolated from pharate pupal molting fluid. The amino acid sequence is similar to those of silkworm, human, mouse, bacterial, and several other β-*N*-acetylglucosaminidases. Two highly conserved regions in the amino acid sequence were found in all members of this family. Southern blot analysis suggested that the number of genes in the *Manduca* genome closely related to the cDNA clone may be as few as one. The β-*N*-acetylglucosaminidase gene is expressed most abundantly in epidermal and gut tissues on days 6 and 7 of fifth instar larvae. Injection of 20-hydroxyecdysone induced expression of the β-*N*-acetylglucosaminidase gene, whereas topical application of the juvenile hormone analog, fenoxycarb, suppressed the inductive effect of molting hormone. Published by Elsevier Science Ltd.

β-*N*-Acetylglucosaminidase Epidermis Integument Cuticle Molting Gut *Manduca sexta* cDNA - Cloning 20-Hydroxyecdysone Juvenile hormone Fenoxycarb Developmental regulation Gene Chitin Tobacco hornworm

INTRODUCTION

Chitin is a linear polymer of β-1,4-linked 2-acetamido-2-deoxy-D-glucopyranoside. It is one of the most unique biochemical constituents found in arthropods and fungi, although evidence has been obtained recently for the occurrence of chitin in vertebrates (Wagner, 1994). In insects, chitin is found in the cuticle of the integument and peritrophic membrane of the midgut. The cuticle provides support and protection through its rigidity and prevents water loss from the body surface. In gut tissues, a cuticular layer also lines both the foregut and hindgut. The peritrophic membrane of the midgut, one function of which is to protect cells lining the midgut from abrasion by food particles, also contains chitin. The insect undergoes periodic shedding or molting of the cuticle to allow for growth and maturation (Kramer and Koga, 1986). During the molting process, the insect secretes a molting fluid that contains hydrolytic enzymes to degrade chitin and protein in the cuticle. Chitin degradation is catalyzed by a two-component chitinolytic enzyme system, chitinase (EC 3.2.1.14) and β-*N*-acetylglucosaminidase (EC 3.2.1.30) (Fukamizo and Kramer, 1985a, b). Chitinase hydrolyzes chitin into oligosaccharides, whereas β-*N*-acetylglucosaminidase further degrades the oligomers to monomers via the nonreducing end.

Because of the critical functions of chitin, it is considered to be a potential target for insect control agents (Kramer *et al.*, 1988; Cohen, 1993), and we are attempting to develop chitinolytic enzymes as biopesticides. Insect pathogens secrete hydrolytic enzymes similar to molting enzymes to penetrate the cuticular barrier (Brandt *et al.*, 1978). Chitinolytic enzymes apparently cause perforation of the peritrophic membrane and exo-

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skeleton, and facilitate the infection caused by pathogens. Chitinase was found to enhance the insecticidal activity of a baculovirus (Shapiro *et al.*, 1987) and the bacterium *Bacillus thuringiensis* (Dubois, 1977). Several strains of the entomopathogenic fungus *Beauveria bassiana* produce β-*N*-acetylglucosaminidase (Bidochka *et al.*, 1993). Certain fungal pathogens, such as *Metarhizium anisopliae*, produce a β-*N*-acetylglucosaminidase and other cuticle-degrading enzymes upon infection of insects (St. Leger *et al.*, 1991).

Chitinolytic enzymes are attractive pest control agents because they can destabilize the cuticle and gut of insects. Since β-*N*-acetylglucosaminidase and chitinase act synergistically *in vitro* to degrade chitin (Fukamizo and Kramer, 1985b), co-expression of these two enzymes *in vivo* may accelerate the degradation of chitin in both the exoskeleton and peritrophic membrane. The genes encoding these enzymes have potential for use in insect pest management, and the long-term goal of this research is to use β-*N*-acetylglucosaminidase and chitinase together as insect control proteins by transferring their genes into baculoviral vectors and plants.

Previously in our laboratory, a full-length cDNA clone encoding a chitinase was isolated from the tobacco hornworm, *Manduca sexta* (Kramer *et al.*, 1993). When infected with a recombinant baculovirus expressing the *Manduca* chitinase gene, larvae of the armyworm, *Spodoptera frugiperda*, died more rapidly than larvae infected with the wild-type virus (Gopalakrishnan *et al.*, 1995). In addition, transgenic tobacco expressing the insect chitinase exhibited resistance to the tobacco budworm, *Heliothis virescens*, in preliminary experiments. As a subsequent study in this project, we report here the cloning of a gene in *M. sexta* for another type of chitinolytic enzyme, β-*N*-acetylglucosaminidase. The expression of this gene was investigated to determine how it is involved in the molting process and how it is regulated by morphogenetic hormones.

MATERIALS AND METHODS

Insect rearing, hormone treatment, and tissue collection

Insect rearing, ligation of larval abdomens, and subsequent hormonal treatments have been described in Corpuz *et al.* (1991). *M. sexta* larvae were staged according to Reinecke *et al.* (1980), and tissues were dissected in Ringer's solution (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$).

For hormone treatments, day 2, fifth instar larvae of *M. sexta* were ligated between the first and second abdominal segments. 20-Hydroxyecdysone was dissolved in 10% isopropanol and injected 2 and 3 days after ligation at a concentration of 7.5 $\mu g\ g^{-1}$ body weight. A juvenoid, fenoxycarb (5 μg in 1 μl ethanol; Maag Agrochemical Co., Vero Beach, FL), was applied topically along the dorsal midline of the abdomen. Pharate pupal molting fluid was collected with a pasteur pipet.

RNA isolation and cDNA library construction

The construction of an *M. sexta* cDNA library has been described previously (Corpuz *et al.*, 1991). Briefly, poly(A^+) RNA was isolated from total RNA by oligo(dT)-cellulose chromatography. After first- and second-strand syntheses, the cDNA was blunt-ended and treated with *Eco*RI methylase. The cDNA was ligated to *Eco*RI linkers and size-fractionated by agarose gel electrophoresis. cDNA with a size greater than 2 kb was ligated into λgt11 arms and packaged into λ-particle using a packaging extract (Promega). The cDNA library was amplified by the plate lysate method (Sambrook *et al.*, 1989).

Library screening

The λgt11 cDNA library was screened with a polyclonal antibody raised against a 62 kDa tobacco hornworm β-*N*-acetylglucosaminidase purified from pharate pupal molting fluid (Koga *et al.*, 1983). The antibody had been purified by DEAE-cellulose chromatography to obtain the IgG fraction, followed by treatment with *Escherichia coli* lysate to reduce nonspecific binding to other proteins. Bacteriophages were plated at a density of 2.5×10^4 plaque-forming units per 150 mm plate in LB medium containing 100 $\mu g\ ml^{-1}$ ampicillin. After incubation for 6–8 h at 37°C, each plate was overlaid with an isopropyl β-D-thiogalactopyranoside (IPTG)-impregnated nitrocellulose membrane and incubated further for 4–6 h. The membranes were blocked with 3% gelatin. After incubation with the β-*N*-acetylglucosaminidase antibody, the positive clones were detected using the Lambda-lift Expression Detection Kit (Bio-Rad).

Subcloning and DNA sequencing

The positive clones detected from the λ-phage library were plaque purified by two more rounds of screening. Single plaques were amplified by the plate lysate method (Sambrook *et al.*, 1989), and the λ DNAs were purified using the Magic Lambda Prep Kit (Promega) and subjected to restriction endonuclease digestion. The DNA fragments were resolved by electrophoresis using 1% agarose gel, purified from the gel using the GeneClean Kit (Bio 101), and ligated into the pBluescript KS^+ vector (Stratagene). The ligation mixture was used to transform *E. coli* JM109 and recombinants were identified by blue/white screening.

The sequences of DNA fragments were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The sequencing reactions were performed with the Sequenase Version 2.0 DNA sequencing kit (U.S. Biochemicals) using [^{35}S]dATP. Plasmids used for sequencing were purified with the Magic Mini-Prep Kit (Promega). Sequencing reactions were applied to a 6% Long Ranger (AT Biochem) or polyacrylamide gel containing 7 M urea. Polymerase chain reaction (PCR) products were sequenced using the *fmol* DNA Sequencing System (Promega).

Database search and sequence analysis

The BLAST computer search program (Altschul *et al.*, 1990) was used to search for similar sequences in protein sequence databases. Sequence alignment and statistical analysis of alignment were performed with the SEQA-LIGN program (Clark, 1991). Hydrophilicity and amino acid composition data were analyzed using MacVector (IBI, Kodak).

Northern and RNA slot blot hybridization

RNA size was determined by Northern blot hybridization. Total RNA was denatured in 2.2 M formaldehyde and separated by electrophoresis using 1.2% agarose containing 0.4 M formaldehyde (Ausubel *et al.*, 1987). The gel was blotted overnight onto a nitrocellulose membrane.

For slot blotting, total RNA from different stages or treatments was denatured and applied to wells of a Minifold II Slot Blotter (Schleicher & Schuell) containing a sheet of nitrocellulose membrane. The membrane was baked and incubated in a prehybridization solution [25 mM potassium phosphate, pH 7.4, 5 × Denhardt's solution, 5 × SSC (standard saline citrate), 100 μg ml^{-1} herring sperm DNA and 50% formamide] for at least 3 h at 42°C and then in hybridization solution (prehybridization solution containing 10% dextran sulfate) overnight at 42°C. The hybridization solution also contained a probe that was random primed and labelled with [^{32}P]dCTP. The probe was purified by passage through a Sepharose CL-6B spin column and denatured by boiling before being added to the hybridization solution. The membrane then was washed with 1 × SSC and 0.1% sodium dodecyl sulfate (SDS) at room temperature followed by washing with 0.2 × SSC, 0.1% SDS at room temperature and exposed to an X-ray film. A laser densitometer (Molecular Dynamics) was used to quantitate the autoradiograms.

Immunoblot and NH_2-terminal sequence analyses

Protein extracts from different tissues and body fluids were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (1970). The proteins then were electroblotted onto nitrocellulose membranes using a semidry blotter (Millipore). Immunoreactive proteins were detected using a polyclonal antibody raised against a tobacco hornworm *β-N*-acetylglucosaminidase from pharate pupal molting fluid (Koga *et al.*, 1983). For NH_2-terminal sequence analyses, a Tricine-buffered SDS–PAGE system and polyvinylidene fluoride membranes were used for protein separation and blotting, respectively (Schagger and von Jagow, 1987).

Southern blot analysis

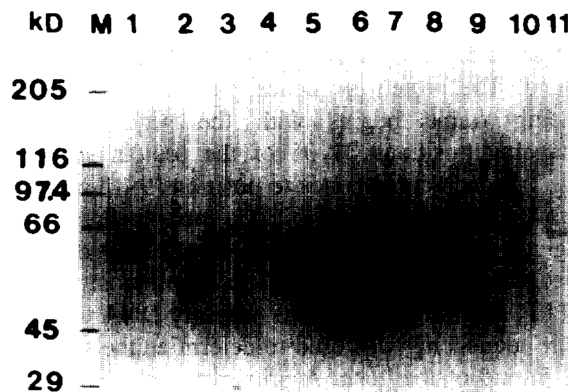
Genomic DNA was digested with different restriction enzymes and separated by agarose gel electrophoresis. The DNA was denatured in 0.5 M NaOH containing 1.5 M NaCl and renatured in 1 M Tris (pH 7.4), 1.5 M NaCl. The protein in the gel then was transferred onto an Immobilon-N membrane (Millipore). The membrane was baked and processed as described under RNA hybridization.

RESULTS

Immunoblot analysis of β-N-*acetylglucosaminidase*

The levels of *β-N*-acetylglucosaminidases in the epidermis and gut tissues during days 5–8 of the fifth larval instar of *M. sexta* were estimated by immunoblot analysis [Fig. 1(A)]. The proteins were detected using a polyclonal antibody against a *β-N*-acetylglucosaminidase purified from pharate pupal molting fluid. In the integument (lanes 2–5), the proteins cross-reacting with *β-N*-acetylglucosaminidase antibody appeared only after day 5 of the fifth instar. One protein with an apparent molecular mass of 62 kDa was detected in the integument. Three proteins with apparent molecular masses of 62, 60, and 55 kDa were detected on days 5 and 6 in the gut tissue of the fifth instar larvae (lanes 6 and 7), whereas only one protein (62 kDa) was detected on days 7 and 8 (lanes 8 and 9). The antibody detected one protein with an apparent molecular mass of 62 kDa in the hemolymph of day 7 and 8 larvae (lanes 10 and 11) and two proteins in the molting fluid with apparent molecular masses of 62 and 55 kDa (lane 1). The NH_2-terminal sequences of the two molting fluid *β-N*-acetylglucosaminidases

A.



B.

1.AEDSP-R-S-EDK
2.GPPGKPNLGWGERTFAIVEVNQAATDY

FIGURE 1. (A) Immunoblot analysis of *β-N*-acetylglucosaminidase expressed during the larval–pupal transformation of *M. sexta*. Protein extracted from different tissues or body fluids was subjected to electrophoresis and electroblotted onto a nitrocellulose membrane. The blot was incubated with antibody raised against the 62 kDa *β-N*-acetylglucosaminidase from pharate pupae of *M. sexta*. Lane 1, molting fluid; lanes 2–5, epidermal tissue from days 5–8 of fifth instar; lanes 6–9, gut tissue from days 5–8 of fifth instar larvae; lanes 10, 11, hemolymph from day 7 and day 8. (B) NH_2–terminal sequences of (1) 62 kDa and (2) 55 kDa *β-N*-acetylglucosaminidases as determined by Edman degradation. –, positions where amino acids could not be identified unambiguously.

(55 kDa and 62 kDa) recognized by the antibody were determined by Edman degradation of protein bands cut from blots obtained after SDS–PAGE [Fig. 1(B)]. A search of the protein sequence databases indicated that the 55 kDa protein had an NH_2-terminal sequence similar to that of *Serratia marcescens* chitinase, whereas the NH_2-terminal sequence of the 62 kDa enzyme was not similar to any of the chitinolytic enzyme sequences listed.

Screening for and sequencing of a β-N-*acetylglucosaminidase cDNA*

A λgt11 cDNA library was constructed from mRNA obtained from the fifth instar larvae (days 5–7). The library was screened with the antibody against the 62 kDa *β-N*-acetylglucosaminidase. Approximately 500,000 recombinant clones from the amplified cDNA library were screened and 42 clones were positive in the first screen. After three rounds of plaque purification, seven putative *β-N*-acetylglucosaminidase clones were picked randomly and analyzed.

The inserts from the recombinant λ-clone DNAs were analyzed by *Eco*RI digestion. Several clones had a 2.4 kb *Eco*RI band. Clone 405, which had *Eco*RI bands of 2.4, 0.35 and 0.2 kb, appeared to have the longest insert. This clone and another clone, 415, with only the 2.4 kb insert, were analyzed by DNA sequencing. The order of the *Eco*RI fragments was determined by sequencing a *Sal*I fragment derived from the λ-clones containing the two internal *Eco*RI sites with synthetic primers designed to bridge the *Eco*RI sites between fragments.

The insert DNA of clone 405 is 2876 nucleotides long and contains an open reading frame extending from the 5′-end. The protein sequence predicted from the open reading frame begins with the sequence SCEDK (spanning positions 31–35 of the protein sequence in Fig. 2), which corresponds to positions 9–13 of the NH_2-terminal sequence of the 62 kDa molting fluid *β-N*-acetylglucosaminidase as determined by Edman degradation [Fig. 1(B)]. Therefore, clone 405 is an incomplete clone.

PCR amplification of total λ-library DNA followed by cycle sequencing was used to determine the sequence of the 5′-end of the gene. A primer complementary to the sequence from positions 61 to 90 of clone 405 and another λgt11 primer (forward or reverse) were used for PCR amplification. The longest PCR product was sequenced. It is 219 nucleotides long and contains an additional 122 nucleotides at the 5′-end followed by the first 90 nucleotides of clone 405.

A composite cDNA sequence of the *β-N*-acetylglucosaminidase gene derived from clone 405 and cycle sequencing is shown in Fig. 2. This sequence contains an open reading frame extending from positions 33 to 1820 and is capable of encoding a protein with 596 amino acids with a molecular mass of 67,882 Da. There is a 1178 nucleotide 3′-untranslated region that is AT-rich and contains several potential polyadenylation signals. The predicted amino acid sequence apparently contains a leader peptide of 22 amino acids, which is followed by the sequence AEDSPWRWSCEDK. This latter sequence matches precisely that of the NH_2-terminal sequence of the 62 kDa *β-N*-acetylglucosaminidase present in *M. sexta* molting fluid. Northern blot analysis revealed that the insert DNA from this clone hybridizes with an RNA of approximately 3.2 kb (Fig. 3), which is consistent with the size of the cDNA (2998 nucleotides).

Sequence analysis

A search of the protein sequence databases for sequences similar to *M. sexta β-N*-acetylglucosaminidase revealed sequence similarities to other *β-N*-acetylglucosaminidases or *β-N*-acetylhexosaminidases. These enzymes include human (Korneluk *et al.*, 1986), murine (Bapat *et al.*, 1988), insect (Nagamatsu *et al.*, 1995), bacterial (Soto-Gil and Zyskind, 1989; Somerville and Colwell, 1993), yeast (Cannon *et al.*, 1994) and other *β-N*-acetylglucosaminidases. The highest similarity was with the enzyme from *Bombyx mori* (Nagamatsu *et al.*, 1995), which has a sequence identity of 71% (Table 1). On the other hand, the identity between the *M. sexta* enzyme and those from human and mouse are 26% and 32%, respectively. Overall, the results of the sequence comparisons suggest that all these proteins are related genetically and share a common ancestor.

Figure 4 shows an alignment of *β-N*-acetylglucosaminidases from several species in two highly similar regions of the amino acid sequences. These regions extend from positions 204 to 253 and from positions 359 to 375 of the *M. sexta* sequence. They contain several conserved, charged, amino acid residues, including four aspartic acids, one glutamic acid, one lysine, two arginines and three histidines, and also several conserved uncharged residues.

The hydrophilicity profile (data not shown) and predicted amino acid composition (data not shown) indicate that the *M. sexta β-N*-acetylglucosaminidase is a hydrophilic protein and that the leader peptide is hydrophobic, as expected. The predicted p*I* is pH 6.0, which is close to the p*I* determined previously by isoelectric focusing (Dziadik-Turner *et al.*, 1981).

Genomic copy number analysis

An estimate of the copy number of the *β-N*-acetylglucosaminidase genes in *M. sexta* was determined by Southern blot hybridization. When the genomic DNA blot was probed first with the insert of clone 415 [Fig. 5(A)], a relatively simple pattern was observed, which indicated the copy number to be low. One major band was observed in the *Eco*RI, *Eco*RV and *Kpn*I digestions, along with one or more minor bands. Three DNA bands were observed after *Sal*I digestion, consistent with the observation that cDNA clone 405 has two internal *Sal*I sites. No internal *Eco*RV or *Kpn*I restriction sites occurred in clone 415.

The presence of multiple bands in the Southern blots could have been due to multiple genes, the presence of

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                                                    GTGACAGTGTGACGTATTACAAAACGTTAACG     32
ATGTGGCTGCGTAGGTTTTACATATACAGCGTGTACATTATCATATTAGCAAAATGTGTTACCACAGCCGAGGACTCGCCATGGCGGTGG    122
M  W  L  R  R  F  Y  I  Y  S  V  Y  I  I  I  L  A  K  C  V  T  T↓ A  E  D  S  P  W  R  W     30
TCGTGCGAAGACAAACGGTGCGTGAAGGTACGCAACGACCCGCAGAACACGGACCCCGTGCTCAGCCTCGAAGCTTGCAAGATGTTCTGC    212
S  C  E  D  K  R  C  V  K  V  R  N  D  P  Q  N  T  D  P  V  L  S  L  E  A  C  K  M  F  C     60
GATGAATACGGTTTGCTGTGGCCGAGGCCGACCGGCGAGACGGATCTGGGAAATTTCTTATCAAAAATAAATATAAACAGCATAGATATA    302
D  E  Y  G  L  L  W  P  R  P  T  G  E  T  D  L  G  N  F  L  S  K  I  N  I  N  S  I  D  I     90
CAAATTCCTAAGCAGGGAAGGAGTGAAAGTCTTTTGAAAGCGGCGGGAAAGAGATTCAAAGATGTGGTGTCACACGCAATACCCAAAGGT    392
Q  I  P  K  Q  G  R  S  E  S  L  L  K  A  A  G  K  R  F  K  D  V  V  S  H  A  I  P  K  G    120
CTTTCACCGAAGGCGACAGGAAAATCTGTCGTCATATACTTAGTCAATGACAATCCTGATATACGAGAATTCTCCCTGGAAATGGACGAG    482
L  S  P  K  A  T  G  K  S  V  V  I  Y  L  V  N  D  N  P  D  I  R  E  F  S  L  E  M  D  E    150
AGCTACGCACTGAGGGTCTCACCGGCATCAAATGAGCGCGTCAATGCCACCATCAGGGCCAACTCGTTCTTCGGCATTCGTCACGGACTC    572
S  Y  A  L  R  V  S  P  A  S  N  E  R  V  N  A  T  I  R  A  N  S  F  F  G  I  R  H  G  L    180
GAGACTCTGTCCCAGCTGATCGTTTATGATGACATTCGAAACCATTTGTTGATCGTAAGAGATGTGACCATAAACGATAAACCCGTGTAC    662
E  T  L  S  Q  L  I  V  Y  D  D  I  R  N  H  L  L  I  V  R  D  V  T  I  N  D  K  P  V  Y    210
CCGTACCGAGGAATTCTATTGGACACTGCCAGGAACTACTACACCATCGACGCCATCAAGAAAACAATTGATGCCATGGCTTCCGCTAAG    752
P  Y  R  G  I  L  L  D  T  A  R  N  Y  Y  T  I  D  A  I  K  K  T  I  D  A  M  A  S  A  K    240
CTGAACACATTCCATTGGCACATCACGGACAGCCAGAGCTTCCCATTCGTCATGGACAAGAGGCCGAACCTCGTCAAGTACGGAGCATAC    842
L  N  T  F  H  W  H  I  T  D  S  Q  S  F  P  F  V  M  D  K  R  P  N  L  V  K  Y  G  A  Y    270
TCTCCAAGTAAAGTGTACACGAAGAAAGCGATCCGCGAGGTGGTGGAGTACGCCCTGGAGCGCGGTGTGCGGTGCTTGCCCGAGTTCGAT    932
S  P  S  K  V  Y  T  K  K  A  I  R  E  V  V  E  Y  A  L  E  R  G  V  R  C  L  P  E  F  D    300
GCGCCCGCGCACGTCGGCGAGGGCTGGCAGGAATCTGATCTCACCGTCTGCTTCAAGGCTGAACCGTGGGCCAAGTACTGTGTGGAGCCA   1022
A  P  A  H  V  G  E  G  W  Q  E  S  D  L  T  V  C  F  K  A  E  P  W  A  K  Y  C  V  E  P    330
CCGTGCGGCCAACTGAACCCCATCAAGGACGAACTCTATGACGTTCTGGAAGATATTTACGTTGAAATGGCCGAGGCGTTCCACTCGACG   1112
P  C  G  Q  L  N  P  I  K  D  E  L  Y  D  V  L  E  D  I  Y  V  E  M  A  E  A  F  H  S  T    360
GACATGTTCCATATGGGAGGAGATGAGGTGAGCGACGCGTGCTGGAATAGTTCAGAAGAAATCCAGCAGTTCATGATTCAGAACCGCTGG   1202
D  M  F  H  M  G  G  D  E  V  S  D  A  C  W  N  S  S  E  E  I  Q  Q  F  M  I  Q  N  R  W    390
GACCTGGACAAGTCTAGCTTCCTCAAGTTGTGGAACTATTTCCAGACAAAGGCAGAGGATAGGGCTTACAAGGCCTTTGGTAAGAATATC   1292
D  L  D  K  S  S  F  L  K  L  W  N  Y  F  Q  T  K  A  E  D  R  A  Y  K  A  F  G  K  N  I    420
CCCTTGGTCATGTGGACGAGCACTCTCACAGACTACACACACGTCGACAAGTTTTTGGACAAGGAAAAATATATCATCCAGGTATGGACT   1382
P  L  V  M  W  T  S  T  L  T  D  Y  T  H  V  D  K  F  L  D  K  E  K  Y  I  I  Q  V  W  T    450
ACAGGCGTAGACCCCCAAATCCAAGGCTTGTTACAGAAAGGCTACAAACTGATTATATCGAACTACGACGCGCTGTACTTTGACTGCGGA   1472
T  G  V  D  P  Q  I  Q  G  L  L  Q  K  G  Y  K  L  I  I  S  N  Y  D  A  L  Y  F  D  C  G    480
TTTGGTGCTTGGGTCGGTTCAGGCAACAACTGGTGTTCACCGTACATCGGATGGCAAAAGGTATACGACAACAGTCCAGCTGTTATGGCA   1562
F  G  A  W  V  G  S  G  N  N  W  C  S  P  Y  I  G  W  Q  K  V  Y  D  N  S  P  A  V  M  A    510
CTATCGTACAGGGACCAAATTCTTGGCGGTGAGGTGGCGCTGTGGTCGGAGCAGGCGGACTCGTCGACGCTAGACGGGCGGCTGTGGCGG   1652
L  S  Y  R  D  Q  I  L  G  G  E  V  A  L  W  S  E  Q  A  D  S  S  T  L  D  G  R  L  W  R    540
CGCCGCCGCCTTCGCCGAGCTGTGTGGGCCGAGCCGCCACCACCTGGCGCGACGCGGAGCAACGCATGCTGCACGTCAGGGAACGCCTTG   1742
R  R  R  L  R  R  A  V  W  A  E  P  P  P  P  G  A  T  R  S  N  A  C  C  T  S  G  N  A  L    570
TGCGCATGGGAATTAAGGCTGAGTCCCTCGAGCCAGAGTGGTGCTACCAAAACGAAGGTTTCTGCTACAATTAAACAGTAGTTTCTCTCC   1832
C  A  W  E  L  R  L  S  P  S  S  Q  S  G  A  T  K  T  K  V  S  A  T  I  K  Q  <             596
ACTGTGTTTAACATCACATATTATAGCTTTTACCCGCACCGTCCGGGATTAATATATATATTCTATACCGATTTATTATAACTAAGAAAC   1922
ATTTATAATTCAAGAATCTTTATCAGTCTGTATTTTGATGTAGCACATGCTATATTATTGCTATTTTTATTATTGGCAAGAATTTGGTGG   2012
TGCCAATAACATTCCTGCCTTGAATGTTGTTAGTATAAAAAATAAATGTAATCTTTTATTAAATCGATTATGTAAAATGTATATTTTATG   2102
TGAAAAATAATATAAGCTTATGTGATTTTTATCGTAAAAATTGAGATTGTAGTTAAGCTAGAGATTGGTATTTTTCGCAATATTTGTAAT   2192
GTGCCATTTGATGACGCATTGCAGAGTATTCAATCTTCTAATCATAATAACAATATAACGCTCTTTCTAAAAGTTAATATCAATGATATC   2282
TATTTATATTTTTAATAATTGTGTTTTTAGCATCTTTGCAATTTATCAATACATTGTATTAAAATCCGATCCGTATAAATGTAAATTTGT   2372
CACACAATTTATGAATATAGGTACAAAAAGTAATTTAAACATATTTTCAGACATACATATCAGAAATATTATCTACATAAAAGGGAAAGT   2462
GAACTGTGTAAATGAATGAAGTTTTTCAAATCATTGGATCTAGAAAAATATTTTAATACGAACTAGTACACATGTTGGACCAGAATGATG   2552
ATTTAGTTAAAAATAAAAAAAGTCCACGACTTACTAACTATTCTGATCTGATCTCTATTAAGATTTTGACATTATATTGACTGTATATAT   2642
TTTTTTTATATATGGCTACAAGCACTGTGTTGTTGTGCTACGCCAATGCTGTATATATTTTATAAGGATGGTAAAAAAGTGGCGTCGAAT   2732
ATGCCATAGATCACTAAATATTTTTTTTTTTTTGGATCAACTGATTTGATTTTCTGACTGTTTTTCCAAATAAATCTAAATAGATTTTGC   2822
CTATGTATATGATGAAGGTTTCGTTGTAAAAGAAATCCAATGACTGATGGATGATTTTTATATAGTAGTTTATACACGTTTTTGTCACAT   2912
TATCGCGGCGTGCTGGTATGTATTATTGCAATGTACTGAGATTGAGGTAATTATTGTGACTACGATCTTATTTTGTAATAAATTTT         2998
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FIGURE 2. Nucleotide and deduced amino acid sequences of *M. sexta* β-*N*-acetylglucosaminidase cDNA. Arrow indicates the position of the mature NH_2-terminus of the 62 kDa β-*N*-acetylglucosaminidase.

introns containing sites for the restriction enzymes used, or incomplete methylation of restriction enzyme sites. To obtain a better estimate of the copy number, the Southern blot was also probed with the two shorter *Eco*RI fragments of clone 405 after stripping away the first probe [Fig. 5(B)]. With the 200 bp *Eco*RI fragment, three DNA bands were observed in the *Eco*RI-digested genomic DNA. One major band and two minor bands were seen in the *Kpn*I digestion. However, only one band each was observed after *Eco*RV and *Sal*I digestions. Probing with the 350 bp *Eco*RI fragment of clone 405 produced three fragments from the *Eco*RI digestion [Fig. 5(C)]. Two fragments were recognized after *Eco*RV digestion. However, only one DNA band was detected after *Kpn*I, *Sal*I, and *Bam*HI digestions. These results suggest that the copy number of β-*N*-acetylglucosaminidase genes in the *Manduca* genome closely related to clone 405 is small and could be as low as one.

Developmental profile of M. sexta β-N-*acetylglucosaminidase*

Expression of the *M. sexta* β-*N*-acetylglucosaminidase gene in epidermis, gut, and fat body was monitored during the larval–pupal transformation by slot blot analysis. The insert DNA of clone 415 was used to probe the expression of the gene (Fig. 6). In the epidermis and gut, low levels of expression were detected from day 0 to day 4 of the fifth instar. Expression increased dramatically starting on day 5 and peaked between days 6 and 7. The level of expression declined on day 8.

In contrast to epidermis and gut, the expression in fat body was quite different. On day 0 of the fifth instar, the level of β-*N*-acetylglucosaminidase expression was relatively high and declined rapidly thereafter. Beyond day 3, the level of the β-*N*-acetylglucosaminidase mRNA in the fat body was low.

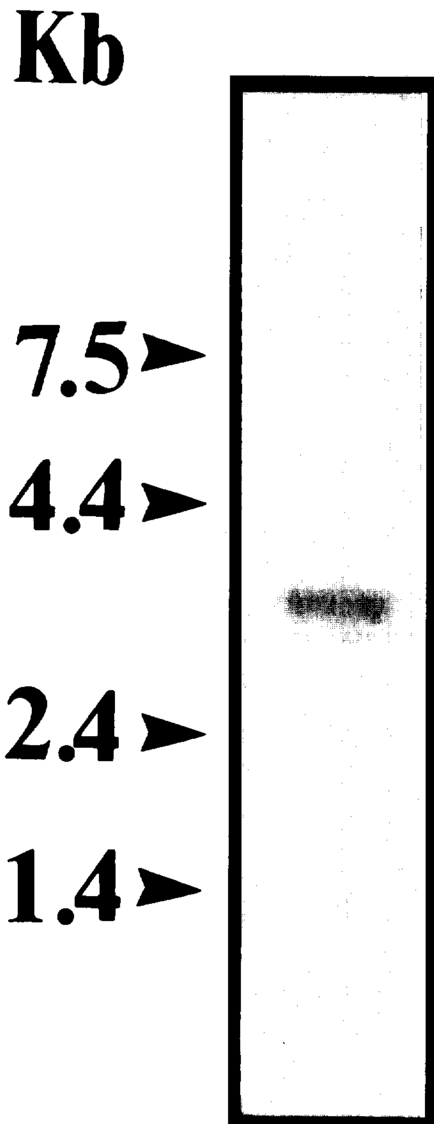

FIGURE 3. Northern blot analysis of total RNA from *M. sexta* for β-*N*-acetylglucosaminidase mRNA. Total RNA isolated from day 5–7 fifth instar larvae of *M. sexta* was resolved in a formamide agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with the 2.4 kb *Eco*RI fragment of clone 415.

Hormonal regulation of M. sexta β-N-*acetylglucosaminidase gene*

The effect of morphogenetic hormones on the expression of the β-*N*-acetylglucosaminidase gene was analyzed after ligation of larval abdomens between the first and second abdominal segments. In the epidermis, the levels of β-*N*-acetylglucosaminidase mRNA increased more than 10-fold within 2 days after treatment with 20-hydroxyecdysone (Fig. 7). Administration of alcohol (as a control) or topical application of fenoxycarb did not induce the transcription of the β-*N*-acetylglucosaminidase gene. When fenoxycarb was administered along with 20-hydroxyecdysone, the induction caused by the latter in the epidermis was diminished greatly.

The induction of β-*N*-acetylglucosaminidase mRNA caused by molting hormone was not as dramatic in gut tissue as in the epidermis (data not shown). Only a 2- to 3-fold increase in the level of the β-*N*-acetylglucosaminidase mRNA occurred in the gut after injection of 20-hydroxyecdysone. Topical application of fenoxycarb by itself had no effect, but it diminished the induction of β-*N*-acetylglucosaminidase caused by 20-hydroxyecdysone.

DISCUSSION

β-*N*-Acetylglucosaminidases and/or β-*N*-acetylhexosaminidases are important carbohydrate-splitting enzymes involved in glycoprotein, polysaccharide, or glycolipid catabolism. In most animals, they are lysosomal enzymes. Deficiency of, or a defect in, these enzymes can be fatal, as in patients suffering from Tay–Sachs disease (Kaback, 1981). In insects, β-*N*-acetylglucosaminidase is mainly a molting enzyme that degrades chitin and chitin-oligosaccharides (Fukamizo and Kramer, 1985a, b). However, the enzyme also may be involved in carbohydrate metabolism other than chitin catabolism (Koga *et al.*, 1982; Altmann *et al.*, 1995).

cDNA clones for β-*N*-acetylglucosaminidase and/or β-*N*-acetylhexosaminidase have been isolated from different species, including human, mouse, silkworm, bacteria and yeast. In this paper, we describe the cloning and sequence analysis of a gene from the tobacco hornworm, *M. sexta*, which encodes this enzyme. The sequence of the encoded protein exhibits similarity to other β-*N*-acetylglucosaminidases, most notably to the recently reported sequence of *B. mori* β-*N*-acetylglucosaminidase (Nagamatsu *et al.*, 1995). The NH_2-terminal sequence from positions 23 to 35 is identical to the NH_2-terminal sequence of a mature 62 kDa β-*N*-acetylglucosaminidase that is present in *M. sexta* molting fluid. Thus, we conclude that this clone encodes the 62 kDa β-*N*-acetylglucosaminidase that is secreted into the molting fluid of *M. sexta* pharate pupae. The predicted size of the mature *M. sexta* β-*N*-acetylglucosaminidase (65 kDa) is comparable to the size estimated from SDS–PAGE analysis (62 kDa).

Considering the size of enzyme, the size of the cDNA is rather large (approximately 3 kb). The large size is due to a long 3′-untranslated region of 1178 nucleotides. This finding is similar to *M. sexta* chitinase cDNA, which has a 757 nucleotide 3′-untranslated region (Kramer *et al.*, 1993). The size of the β-*N*-acetylglucosaminidase mRNA (3.2 kb) is in agreement with the size of the β-*N*-acetylglucosaminidase cDNA clone. The reason why we did not detect the full-length cDNA in the library is unclear, but probably the full-length clone in our library is present only in very low abundance.

On the basis of sequence similarity, β-*N*-acetylglucosaminidases fall into one family of glycosylhydrolases (Henrissat, 1991). These enzymes utilize substrates similar to those used by endochitinases and lysozymes. A substrate for lysozyme is the bacterial cell wall that consists of alternating 2-acetamido-2-deoxy-D-glucopyrano-

TABLE 1. Analysis of the similarities in amino acid sequences between *M. sexta* β-*N*-acetylglucosaminidase and enzymes from other species

Species	Z-value	Per cent identical residues
Bombyx mori	178.79	71
Dictyostelium discoideum	27.02	34
Mus musculus	25.22	32
Felis catus	29.56	31
Vibrio vulnificus	9.15	30
Homo sapiens	23.84	26
Candida albicans	27.19	25
Porphyromonas gingivalis	13.79	24
Vibrio harveyi	9.39	22
Entamoeba histolytica	11.63	14

Sequences were analyzed by Seqalign subprogram GPALIGN 2 (Clark, 1991). The Z-value was calculated based on 50 random sequences with an identity matrix used for scoring and is defined as the score of the actual sequences minus the average score of the alignments of the pairs of randomized sequences, divided by the standard deviation of the scores from the randomizations. *Z* is the number of standard deviations that a score lies above the mean of the distribution.

REGION I

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 1:(309) I N D E P R L D Y R G M H M D V S R N F H S K E L V F R F L D Q M A A Y K M N K F H F H L A D D E G (358)
 2:(326) I K D A P R F D Y R G V M V D V A R N F H S K D A I L A T L D Q M A A Y K M N K L H L H L T D D E G (375)
 3:(135) I S D A P R F K W R G L M V D P S R N P L S P L M F K R I I D T L A S V K A N V L H I H L S D A Q T (184)
 4:(160) I S D F P N F K H R G L M I D S G R N F L T V D S I L E Q I D I M A L S K M N S L H W H L A D S Q S (209)
 5:(136) I I D S P R F P H R G I L I D T A R H F L P V K S I L K T L D A M A F N K F N V L H W H I V D D Q S (185)
 6:(173) I A D S P R F P H R G I L I D T S R H L L P V K T I F K T L D A M A F N K F N V L H W H I V D D Q S (222)
 7:(161) I E D F P R F P H R G L L L D T S R H Y L P L S S I L D T L D V M A Y N K L N V F H W H L V D D P S (210)
 8:(149) I S D S P R Y P W R G F M V D S A R H Y I P K N M I L H M I D S L G F S K F N T L H W H M V D A V A (198)
 9:(164) I K D E P A F G Y R G F M L D V C R H F L S V E D I K K H I D I M A M F K I N R F H W H L T E D Q A (213)
10:(205) I K D R P V Y P Y R G I L L D T A R N F Y S I D S I K R T I D A M A A V K L N T F H W H I T D S Q S (254)
11:(204) I N D K P V Y P Y R G I L L D T A R N Y Y T I D A I K K T I D A M A S A K L N T F H W H I T D S Q S (253)
         * - * + * - + - + * * + + + * + - * + + + + + + - + + + - + * + + + - - * - * - + * + * + + + + + +
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REGION II

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 1:(509) P L T D Y H I G A D E T A G A W G (525)
 2:(527) P L T T W H F G G D E A K N I K L (543)
 3:(291) G T D Y V H V G G D E V W T S G W (307)
 4:(314) I D D V F H V G N D E L Q E K C Y (330)
 5:(287) P D H F V H L G G D E V E F Q C W (303)
 6:(327) P D Q F I H L G G D E V E F Q C W (343)
 7:(274) P D F Y L H L G G D E V D F T C W (290)
 8:(298) I D N Y F H T G G D E L V T G C W (314)
 9:(325) P G T Y F H I G G D E C P K D R W (341)
10:(360) S T D M F H M G G D E V S E R C W (376)
11:(359) S T D M F H M G G D E V S D A C W (375)
         + - + + + * + * + * * - - - - - +
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FIGURE 4. Comparison of regions of high similarity in amino acid sequences of β-*N*-acetylglucosaminidases. 1, *Vibrio vulnificus*; 2, *Vibrio harveyi*; 3, *Entamoeba histolytica*; 4, *Candida albicans*; 5, *Felis catus*; 6, *Mus musculus* (β-chain); 7, *Homo sapiens* (α-chain); 8, *Dictyostelium discoideum*; 9, *Porphyromonas gingivalis*; 10, *Bombyx mori*; 11, *Manduca sexta*. ★, position is identical in all sequences; +, position has a positive contribution to the score; –, position has a negative contribution to the score.

side and *N*-acetylmuramic acid residues. Endochitinases, β-*N*-acetylglucosaminidases, and some of the lysozymes can also degrade chitin and its oligomers. Similarities in the three-dimensional structures of hen egg-white lysozyme and barley endochitinase were reported (Holm and Sander, 1994), which led to the identification of Glu67 as a catalytic residue in barley endochitinase, corresponding to Asp52 in chicken lysozyme. Likewise, the similarity in catalytic mechanisms between β-*N*-acetylglucosaminidases and endochitinases suggests that the former family of enzymes has a three-dimensional structure similar to that of the latter and also of lysozymes. Two regions of high sequence similarity, which were identified by sequence comparisons of proteins from different species, may be critical for catalysis and/or substrate binding. The function(s) of these two regions can be studied by site-directed mutagenesis in the future. Of particular interest are the acidic amino acid residues in these regions, because these residues are involved in both catalytic and substrate-binding contacts in the active sites of many other glycosylhydrolases.

Southern blot analysis revealed that the copy number of genes in *M. sexta* related to β-*N*-acetylglucosaminidase is small. This conclusion is based upon the small number of bands in Southern blots probed with the shortest DNA fragments, which should have avoided compli-

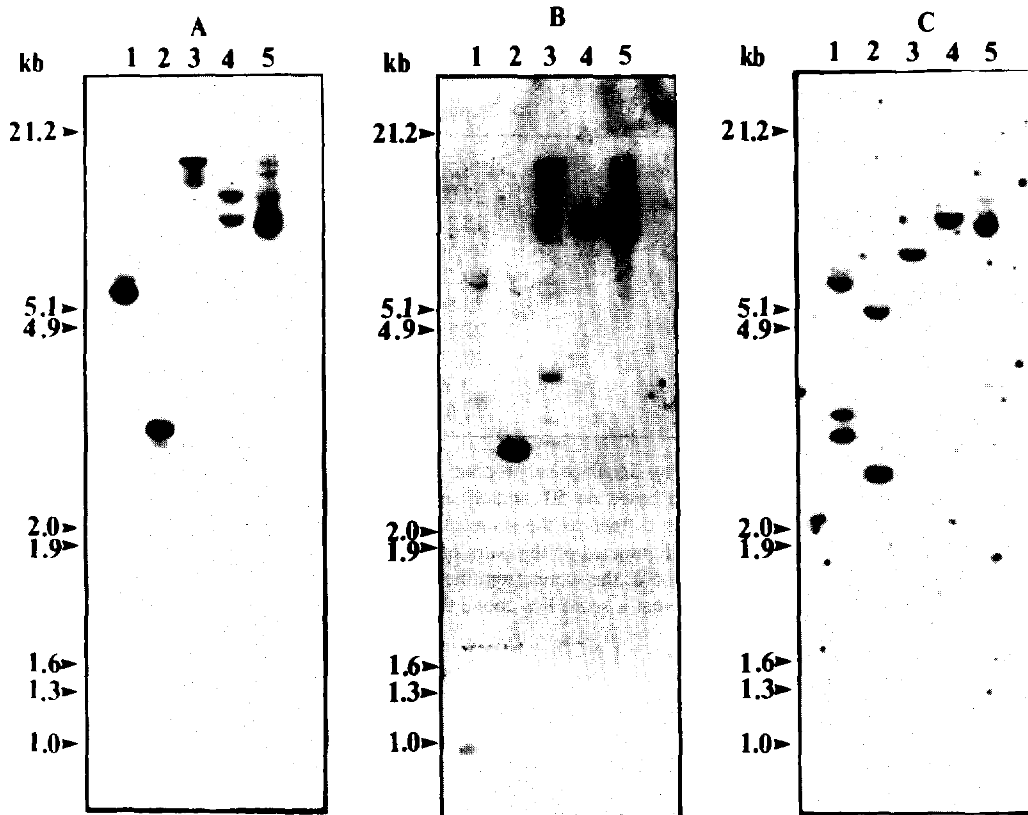

FIGURE 5. Southern blot analysis of *M. sexta* DNA for β-*N*-acetylglucosaminidase genes. Genomic DNA was digested with different restriction enzymes and subjected to gel electrophoresis using 1% agarose followed by capillary transfer onto an Immobilon-N membrane. Lane 1, *Eco*RI; lane 2, *Eco*RV; lane 3, *Kpn*I; lane 4, *Sal*I; lane 5, *Bam*HI. (A) Membrane probed with ^{32}P-labeled clone 415 2.4 kb *Eco*RI fragment. (B) Membrane probed with ^{32}P-labeled clone 405 200 bp *Eco*RI fragment. (C) Membrane probed with ^{32}P-labeled clone 405 350 bp *Eco*RI fragment.

cations from the presence of introns. The finding that digestions with three restriction enzymes resulted in the detection of a single band suggests a single copy of the gene. Even though digestion with two other enzymes (*Eco*RI and *Eco*RV) yielded two or three bands, the minor bands in these digests were probably due to partial methylation of flanking sites for the enzymes. However, immunoblot analysis demonstrated that two proteins were recognized by the β-*N*-acetylglucosaminidase antibody. Analysis of the NH_2-termini of these two proteins revealed that they have different NH_2-terminal sequences. The sequence of the 62 kDa molting fluid protein corresponds to the cDNA characterized in this study. The second 55 kDa enzyme is not a truncated form of the 62 kDa protein because the NH_2-terminal sequence of the latter, GPPGKPNLGWGERTFAIVEVNQAATDY, does not correspond to any region within the predicted sequence of the protein encoded by the β-*N*-acetylglucosaminidase cDNA clone. On the other hand, the NH_2-terminal sequence of the 55 kDa protein resembles that of *Serratia* chitinase (Harpster and Dunsmuir, 1989). Furthermore, the 62 kDa protein has no significant sequence similarity to the *Serratia* enzyme or any other chitinolytic enzymes except β-*N*-acetylglucosaminidases. These observations suggest that the two molting fluid β-*N*-acetylglucosaminidases may belong to different families, in spite of the fact that they share a common epitope recognized by a polyclonal antibody. Based on the sequence differences, the DNA probe using clone 405 may not cross-react with the second β-*N*-acetylglucosaminidase gene.

Insect molting and morphogenesis are controlled by specific hormones. The levels of β-*N*-acetylglucosaminidase in the epidermis and gut during days 5–8 of the fifth instar larvae appear to parallel the developmental titers of ecdysone (Riddiford, 1994). In the epidermis, both chitinase and β-*N*-acetylglucosaminidase mRNAs are expressed during molting. However, the expression of these two enzyme genes is somewhat different. Chitinase mRNA disappears by day 8 (Kramer *et al.*, 1993), whereas β-*N*-acetylglucosaminidase is still present at a relatively high level at that time. Nevertheless, the overlap of the periods of expression of the two enzymes supports the notion that chitinase and β-*N*-acetylglucosaminidase act in tandem to catabolize cuticular chitin (Fukamizo and Kramer, 1987). β-*N*-Acetylglucosaminidase is also expressed in the fat body early in the fifth instar, which may reflect residual mRNA carried over from the fourth instar. The significance of the expression of β-*N*-acetylglucosaminidase in the fat body is unclear.

The expression of the β-*N*-acetylglucosaminidase gene in epidermal tissue of ligated larvae was stimulated gre-

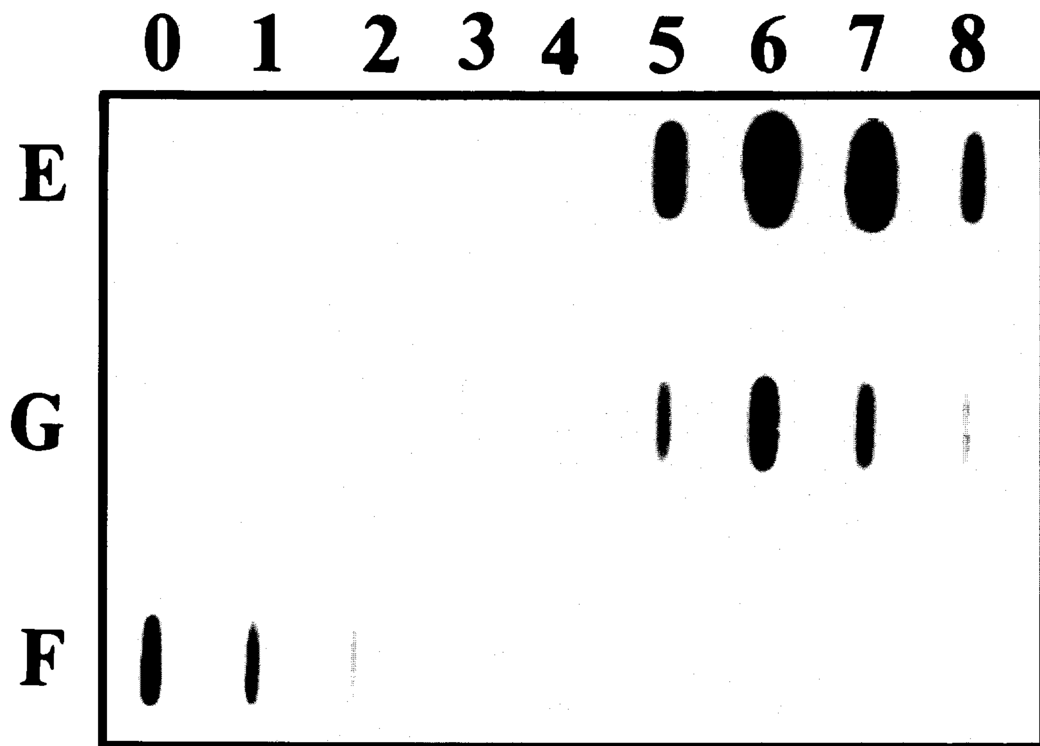

FIGURE 6. Developmental profile and tissue distribution of *M. sexta* β-*N*-acetylglucosaminidase mRNA. Total RNA from epidermis (E), gut (G), and fat body (F) tissues of *M. sexta* at different stages (days) of the fifth larval instar was applied to each well of a slot blotter. The membrane was probed with ^{32}P-labeled 2.4 kb *Eco*RI restriction enzyme fragment from clone 415.

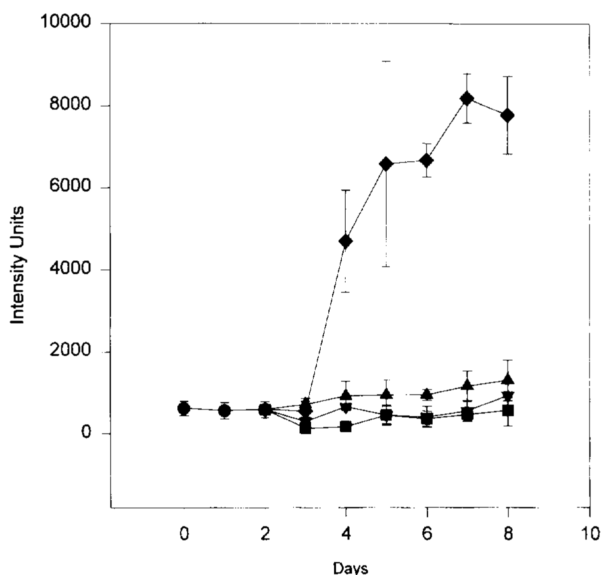

FIGURE 7. Effect of hormone treatments on *M. sexta* β-*N*-acetylglucosaminidase gene expression in the epidermis. Tobacco hornworms were ligated between the first and second abdominal segments. 20-Hydroxyecdysone was injected, and fenoxycarb was applied topically. Total RNA was estimated by slot blot analysis, and the autoradiograms were quantitated by densitometry. ●, before hormone treatment; ■, control treated with isopropanol only; ▼, treated with fenoxycarb; ◆, treated with 20-hydroxyecdysone; ▲, treated with fenoxycarb and 20-hydroxyecdysone.

atly by injection of 20-hydroxyecdysone. This result is consistent with previous reports that ecdysteroids induce chitinolytic enzyme expression (Kimura, 1973; Fukamizo and Kramer, 1987; Kramer *et al.*, 1993). The molting hormone-induced expression of β-*N*-acetylglucosaminidase in *M. sexta* epidermis is suppressed by a juvenoid. In *Ephestia cautella*, chitinolytic enzyme activity and ecdysteroid titer are affected similarly by a juvenoid (Spindler-Barth *et al.*, 1986).

The cDNA clone of the *M. sexta* β-*N*-acetylglucosaminidase gene characterized in this study will be used in the future to acquire a full-length cDNA. The full-length clone will be used to transform either *E. coli* or a baculovirus to obtain relatively large amounts of the recombinant protein, which will be bioassayed alone and in combination with the other chitinolytic enzyme, chitinase, to determine whether insect growth and development will be affected adversely.

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