

# Sequence of a cDNA and Expression of the Gene Encoding Epidermal and Gut Chitinases of *Manduca sexta*\*

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Insects use chitinolytic enzymes to digest chitin in the exoskeleton during the molting process. We have isolated and sequenced a chitinase-encoding cDNA from the tobacco hornworm, *Manduca sexta*, compared its sequence with genes encoding chitinolytic enzymes from other sources, and studied chitinase gene expression and hormonal regulation during the larval-pupal transformation. The insert DNA in this clone is 2452 nucleotides long with an open reading frame of 1662 nucleotides that encodes a protein of 554 amino acids with a molecular weight of 62 kDa. Several regions of the amino acid sequence in this protein are similar to sequences in yeast, cucumber and bacterial endo- $\beta$ -*N*-acetylglucosaminidases. Hybrid-selection of mRNA and *in vitro* translation yielded an immunoreactive protein with an apparent molecular mass of 75 kDa, which is similar to the size of a chitinase present in pharate pupal molting fluid. Southern blot analysis indicated that one or two genes related to the cDNA clone are encoding chitinases in the *Manduca* genome. The major tissues expressing chitinase genes were the epidermis and gut with mRNA levels highest on *c.* days 5-7 during the fifth larval instar. Injection of 20-hydroxyecdysone into ligated fifth instar abdomens caused about a 10-fold increase in mRNA levels in both epidermis and gut, and topical application of the juvenile hormone mimic, fenoxycarb, suppressed the ecdysteroid-induced accumulation of chitinase RNA.

Chitinase Endo- $\beta$ -*N*-acetylglucosaminidase Epidermis Integument Cuticle Molting Gut *Manduca sexta* cDNA 20-Hydroxyecdysone Juvenile hormone Fenoxycarb Developmental regulation Gene

## INTRODUCTION

Insects undergo periodic ecdysis or shedding of their exoskeleton and synthesis of a new one to allow for continued growth and development. Digestion of the unsclerotized layers of the cuticular exoskeleton is necessary prior to ecdysis and is catalyzed by several hydrolytic enzymes elaborated by epidermal and gut tissues that digest chitin and protein, leaving the more highly sclerotized portions behind to be shed as an exuvium. Enzymatic degradation of the cuticle between apolysis and ecdysis was first established by Passonneau and Williams (1953), who studied the molting fluid of pharate adult cecropia moths. Some of the chitinolytic

and proteolytic enzymes in molting fluid of the tobacco hornworm, *Manduca sexta*, were characterized in our laboratory (Koga *et al.*, 1982, 1983a; Brookhart and Kramer, 1990). Now we have begun to clone and characterize the genes for hydrolytic enzymes found in the molting fluid of *M. sexta* in order to help understand how the expression of these enzymes is precisely regulated by endocrine and developmental events during the ecdysial process. Cloning of the genes involved in chitinolysis serves as a first step towards introduction of these genes into microbes and plants for utilization of the enzymes as control agents in the management of insect pests and plant pathogens.

Two types of enzymes from pharate pupal molting fluid of *M. sexta* act in tandem to digest chitin. The insoluble  $\beta$ (1-4)-linked polysaccharide is first cleaved by endo-splitting chitinases and then the soluble oligosaccharide products are subsequently hydrolyzed to 2-acetamido-2-deoxyglucopyranoside (GlcNAc) by exocleaving  $\beta$ -*N*-acetylglucosaminidases (Kramer *et al.*, 1985; Kramer and Koga, 1986). The former enzyme is rate-limiting for chitin degradation and its levels are elevated in response to 20-hydroxyecdysone (20-HE)

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administration, whereas co-administration of a juvenile hormone mimic with 20-HE suppresses the increase in chitinase activity (Fukamizo and Kramer, 1987).

In this paper, we describe the cloning of a cDNA encoding a chitinase-like protein from *M. sexta*, its nucleotide sequence, and the deduced amino acid sequence of the encoded protein, as well as the developmental profile and effects of morphogenetic hormones on the expression of chitinase genes during the larva-pupal transformation. Two regions of the amino acid sequence are very similar to regions in bacterial and plant endo- $\beta$ -*N*-acetylglucosaminidases which have been proposed to be part of a catalytic domain (Henrissat, 1990). Chitinase gene expression was observed in epidermal and gut tissues, but not in fat body and muscle, during the larval-pupal transformation of *M. sexta*. Expression was stimulated at the level of transcription by 20-HE and inhibited by the juvenile hormone mimic, fenoxycarb.

## MATERIALS AND METHODS

### *Insects and tissue collection*

Insect rearing, hormone treatments, ligation, and tissue collection were carried out according to Corpuz *et al.* (1991). Ligated abdomens were injected through a proleg with  $7.5 \mu\text{g g}^{-1}$  body weight 20-hydroxyecdysone (Sigma Chemical Co., St Louis, Mo) dissolved in 10% isopropanol at day 2 and day 3 after ligation (Fukamizo and Kramer, 1987). A juvenile hormone mimic, fenoxycarb (Maag Agrochemical Co., Vero Beach, Fla), was applied topically ( $5 \mu\text{g}$  in  $1 \mu\text{l}$  ethanol) along the dorsal midline. Whole larvae, fifth instar integument, fat body, muscle, and gut were quick-frozen in liquid nitrogen, stored at  $-70^\circ\text{C}$ , and thawed directly into 4 M guanidine isothiocyanate buffer for RNA isolation. Molting fluid was collected in a microcapillary tube from day 8 fifth instar larvae through a puncture in the dorsal abdominal horn or a proleg, transferred to a plastic microcentrifuge tube containing phenylthiourea to inhibit phenoloxidase activity, and stored at  $-20^\circ\text{C}$ .

### *RNA and DNA isolation*

Total RNA was isolated from homogenized tissue by the method of Chirgwin *et al.* (1979) as modified by Ausubel *et al.* (1987). Poly(A) + RNA was isolated from total RNA by oligo(dT)-cellulose column chromatography (Aviv and Leder, 1972). High molecular weight genomic DNA was prepared from day 4 fifth instar larvae as described by Corpuz *et al.* (1991).

### *cDNA library construction*

Poly(A) + RNA ( $40 \mu\text{g}$ ) from whole larvae was used for cDNA synthesis using standard protocols (Ausubel *et al.*, 1987). Briefly, after reverse transcription and second strand synthesis, the double-stranded cDNA product was blunt-ended with T<sub>4</sub> DNA polymerase and treated with EcoRI methylase to protect EcoRI restric-

tion sites within the cDNA. The cDNA was then ligated to a 150-fold molar excess of EcoRI linkers, digested with EcoRI, and size-selected by agarose gel electrophoresis to obtain molecules with length  $> 1600$  bp. Following ligation of the cDNA to EcoRI-digested  $\lambda$ gt11 arms, the ligated DNA was packaged into phage particles, which were introduced into *E. coli* Y1090r<sup>-</sup>. The cDNA library was amplified on LB plates containing  $50 \mu\text{g ml}^{-1}$  ampicillin and  $10 \text{ mM MgSO}_4$ . The library contained  $c. 1.2 \times 10^6$  plaque-forming units in the library with  $> 90\%$  recombinants.

### *cDNA library screening*

The cDNA library was screened using the lambda-lift expression detection kit (Bio-Rad Lab.). Plating was done at high density ( $30\text{--}50 \times 10^3$  plaques per 150 mm plate), and initial screening was carried out with a polyclonal antibody raised against *M. sexta* chitinase (Koga *et al.*, 1983b). About 60 strongly positive plaques were purified by rescreening two or three rounds with the same antibody probe at a low plating density (200–300 plaques per 100 mm plate). Phage DNA was isolated from positive plaques (Ausubel *et al.*, 1987) and subcloned into Bluescript (Stratagene) or M13 vector for further study.

### *Southern hybridization*

Genomic DNA was digested with a 10-fold excess of restriction enzymes SmaI, HindIII, EcoRV, and BamHI (Promega); subjected to agarose gel electrophoresis; and transferred onto nitrocellulose. The filters were prehybridized at  $65^\circ\text{C}$  using 2X SSC (SSC = 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7). After hybridization for 16 h according to the procedure of Maniatis *et al.* (1982), the membranes were washed with 2X SSC for 30 min at room temperature, followed by 30 min washes at 50 and  $65^\circ\text{C}$ . The <sup>32</sup>P-labeled insert DNA probe (clone 10, see later) had a specific activity of  $6 \times 10^8$  cpm  $\mu\text{g}^{-1}$  and was used at  $1 \times 10^6$  cpm  $\text{ml}^{-1}$  of hybridization solution.

### *Northern and RNA slot blot hybridization*

RNA analysis by Northern and slot blot hybridization was performed under medium stringency conditions and quantitated essentially as described by Corpuz *et al.* (1991). Hybridization and wash conditions were the same as those described above for Southern blots. Quantitation of slots blots was by densitometry.

### *Hybrid selection, in vitro translation and immunoprecipitation*

Several of the clones detected by the chitinase antibody contained a common 1.8 kb EcoRI fragment and a second EcoRI fragment of variable length. Clone 10, which contained only the 1.8 kb fragment, was chosen for hybrid selection experiments because it was shown to contain the entire chitinase coding region by DNA sequencing (see later). The insert DNA from this clone

was subcloned into M13 mp 18 according to Maniatis *et al.* (1982). Each of the two single-stranded recombinant subclones in both orientations (15 µg DNA) was bound to a 2 cm<sup>2</sup> diazophenylthioether cellulose paper. Filters were prehybridized according to the method of Chandra *et al.* (1985) and hybridized to 1 or 2 mg total RNA from day 6 fifth instar larvae in 50% deionized formamide, 0.4 M NaCl, 0.2% SDS, 20 mM Pipes (pH 6.4) and 200 µg ml<sup>-1</sup> yeast tRNA at 40°C for 16–18 h (Miller *et al.*, 1983). Filters were washed ten times with a buffer containing 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, and 0.2% SDS at 65°C and then three times with the same buffer lacking SDS. RNA was eluted in 0.3 ml sterile water containing yeast tRNA and precipitated by addition of ethanol. The RNA was solubilized and translated using a rabbit reticulocyte lysate *in vitro* translation system (Promega) and L-[<sup>35</sup>S]methionine (1000 Ci mmol<sup>-1</sup>; New England Nuclear) as the labeled amino acid. Translation products were analyzed on 8% SDS-polyacrylamide gels (Laemmli, 1970). The stained gels were treated with Enhance (New England Nuclear), dried, and exposed to Kodak XAR-5 X-ray film.

The procedure of Clemens (1984) was used for immunoprecipitation of chitinase immunoreactive protein. Molting fluid (0.1 ml) or translation products were incubated with 0.01 ml preimmune serum, and the nonspecific complexes were removed by precipitation with 0.05 ml Protein A-Sepharose beads (Pharmacia). After centrifugation, the supernatant was incubated with 0.02 ml chitinase antiserum or 0.01 ml preimmune serum for 12–16 h at 4°C, and the immune complexes were precipitated by 0.05 ml Protein A-Sepharose beads. Following centrifugation, the pellet was washed six times with immunoprecipitation buffer containing 1 mg ml<sup>-1</sup> methionine. The washed pellet was heated at 100°C for 3 min in 0.04 ml SDS-PAGE sample buffer and stored at -20°C or used immediately for electrophoresis.

#### DNA sequencing and data base search

From a total of 67 chitinase related cDNA clones, five that ranged in insert size from 1.8 to 2.5 kb were chosen for sequence analysis. Clone 10 had a 1.8 kb insert, and the longest insert (2.45 kb) was found in clone 201. All clones with an insert longer than 1.8 kb had an internal EcoRI restriction site. Furthermore, all clones contained an internal BamHI site within the 1.8 kb EcoRI fragment. This site was utilized for generating subclones. Inserts from all five clones were subcloned into pBlue-script and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using [<sup>35</sup>S]dATP (Biggin *et al.*, 1983) and Sequenase version 2.0 (Tabor and Richardson, 1987). Synthetic primers (complementary to either strand) were used to complete the sequencing. Sequencing reaction products were analyzed on 6% polyacrylamide gels in 7 M urea.

The protein and nucleic acid data bases of the Protein Identification Resource of NBRF at Georgetown University were searched for related sequences. Sequence

alignment and statistical analysis of the alignment scores were carried out with the 'SEQALIGN' program developed by Kirk Clark and Gerald Reeck of Kansas State University (Clark, 1991).

## RESULTS

### Isolation of chitinase cDNA clones

Because chitinolytic enzyme activity in *M. sexta* is maximal around the time of apolysis, when molting fluid fills the space between the old cuticle being digested and the new cuticle being synthesized (Kramer *et al.*, 1985), we constructed a cDNA library in λgt11 phage starting with poly(A) + RNA extracted from a mixture of day 5–day 7 fifth instar larvae that had not yet undergone apolysis. This cDNA library was screened with antibody raised against *M. sexta* chitinase (Koga *et al.*, 1983b). 67 positive plaques, from a total of 2 × 10<sup>5</sup> screened, were purified by replating at low density and further screened with purified chitinase antibody. All 67 clones cross-hybridized to one another, and one of the larger clones, a 1.8 kb clone designated as clone 10, was selected for use as a probe in further studies. A later screening of the same cDNA library for larger cross-hybridizing clones resulted in the isolation of clone 201, which had the longest insert (2.45 kb). These two clones and three others were used for sequencing analysis.

### Clone 10 codes for a 2.6 kb transcript and a 75 kDa protein

Northern blot analyses of epidermal and gut RNAs from day 6 fifth instar *M. sexta* larvae using clone 10 as the probe detected transcripts c. 2600 nucleotide long in both tissues (Fig. 1). To determine the size of *M. sexta* proteins corresponding to these RNAs, insert DNA from clone 10 was subcloned into M13 in both orientations and used to hybrid-select mRNA, which was then translated *in vitro* with [<sup>35</sup>S]methionine as the radioactive amino acid (Fig. 2). Total RNA translation products included several protein bands as expected (lane 1). With one orientation of the clone, no mRNA hybridized since protein products were not detected by *in vitro* translation (lanes 2 and 3). With the opposite orientation clone (lanes 4 and 5), a protein with an apparent molecular weight of 75 kDa was detected after SDS-PAGE and autoradiography. This 75 kDa protein was immunoprecipitated by the chitinase antibody from translations of total RNA and hybrid-selected RNA (lanes 7 and 10). No immunoreactive protein band was found when preimmune serum was used (lane 9). The 75 kDa immunoreactive protein has the same size as one of the chitinases isolated from *M. sexta* pharate pupal molting fluid (Koga *et al.*, 1983a).

### Chitinase is encoded by a small multicopy gene family

The number of chitinase genes in *M. sexta* genomic DNA was estimated by Southern blot analysis (Fig. 3) after digestions with SmaI (lane 1), HindIII (lane 2),

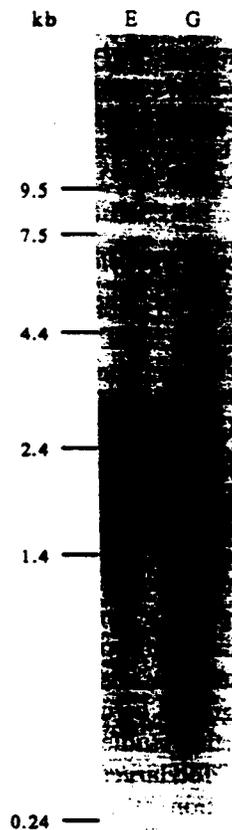


FIGURE 1. Autoradiogram of northern blot of epidermis (E) and gut (G) mRNA (2.5  $\mu$ g) of *M. sexta* day 6 fifth instar larvae hybridized to  $^{32}$ P-labeled insert DNA from clone 10. RNA size markers are indicated in kilobases (kb).

EcoRV (lane 3), and BamHI (lane 4) restriction endonucleases and probing with  $^{32}$ P-labeled insert DNA from clone 10. When medium stringency washing conditions (65°C, 2x SSC) were employed, three to five hybridizing bands with different mobilities resulted from the four restriction enzyme digests. The clone 10 cDNA insert had a single site for BamHI, but no sites for SmaI, HindIII, or EcoRV. The same blot was reprobed with one or the other of the two BamHI fragments derived from clone 10 cDNA insert. Of the four bands seen in the BamHI digest (lane 4), only the two upper bands were detected by one BamHI fragment probe, whereas only the lower two bands were detected by the other BamHI fragment probe (data not shown). These results indicated that two of the four bands in the BamHI digest were due to the presence of internal BamHI sites within the chitinase gene and suggested the presence of either one or two genes in the *M. sexta* genome related to clone 10.

#### *Nucleotide sequence of clone 201 and deduced amino acid sequence of its encoded chitinase*

The complete nucleotide sequence of the insert in clone 201 is shown in Fig. 4. The insert from this clone is 2452 nucleotides long and contains an internal EcoRI site at position 1804. Clone 10 begins at the same position as clone 201 but ends at this EcoRI site. The

other three clones that we have sequenced differ from clone 201 only with respect to the start and end positions of the insert and, therefore, are truncated forms of clone 201. No sequence heterogeneity was found among the five clones. Clone 201 contains an open reading frame (ORF) stretching from the ATG codon 34 nucleotides downstream from the 5'-end to position 1695 (Fig. 4). This ORF of 1662 nucleotides encodes a protein of 554 amino acids that has a molecular weight of 62,210 Da. The 3'-untranslated region of 757 nucleotides is AT-rich and contains several putative polyadenylation signals. The N-terminal sequence of the encoded protein contains numerous hydrophobic residues characteristic of a leader peptide.

A search of the data bases of the Protein Identification Resource, NBRF, revealed that the protein encoded by this clone is related to the endo- $\beta$ -*N*-acetylglucosaminidase (endo H) from *Streptomyces plicatus* (Robbins *et al.*, 1984). Even though the number of amino acid identities was not very high, (57 identities, representing 21% of the aligned positions), the sequences were indeed clearly similar ( $z$  value = 24.4, data not shown). The amino acid sequence includes two regions that are highly conserved in yeast, cucumber, and bacterial chitinases. The conserved regions are located from residues 97 to 111 (region I) and from 139 to 148 (region II) in the *M. sexta* chitinase (Fig. 5). The latter region is more similar than the former, and in particular, it contains three aspartic acid residues and one glutamic acid, all or some of which have been suggested to participate in the binding of substrate or enzymatic hydrolysis of glycosidic bonds (Henrissat, 1990). In addition to these two regions of highest similarity, many threonines and serines occur between positions 398 and 453 in the C-terminal region of this protein (Fig. 4). Likewise, a threonine/serine rich domain is found near the C-terminus of yeast chitinase (Kuranda and Robbins, 1991).

#### *Tissue-specific expression of M. sexta chitinase genes*

To determine whether the *M. sexta* chitinase genes exhibit tissue-specific expression, slot blot analysis was used to quantitate the amount of chitinase mRNA in selected tissues. Total RNA was prepared from muscle, epidermis, fat body, and gut tissues of day 6 fifth instar larvae and 5  $\mu$ g amounts of total RNA were probed with the insert from clone 10 (Fig. 6). On day 6, gut contained the highest concentration of chitinase mRNA on a per microgram RNA basis, followed by epidermis. Muscle contained a very low amount of chitinase mRNA, whereas fat body contained none. These results indicated that on day 6 of the fifth instar, gut and epidermis are the major tissues that express chitinase genes.

#### *Developmental changes in chitinase mRNA levels*

Changes in the levels of chitinase transcripts in epidermal and gut tissues throughout development of fifth instar larvae were determined by slot blot analyses. For this purpose, duplicate blots of total RNA from day 0

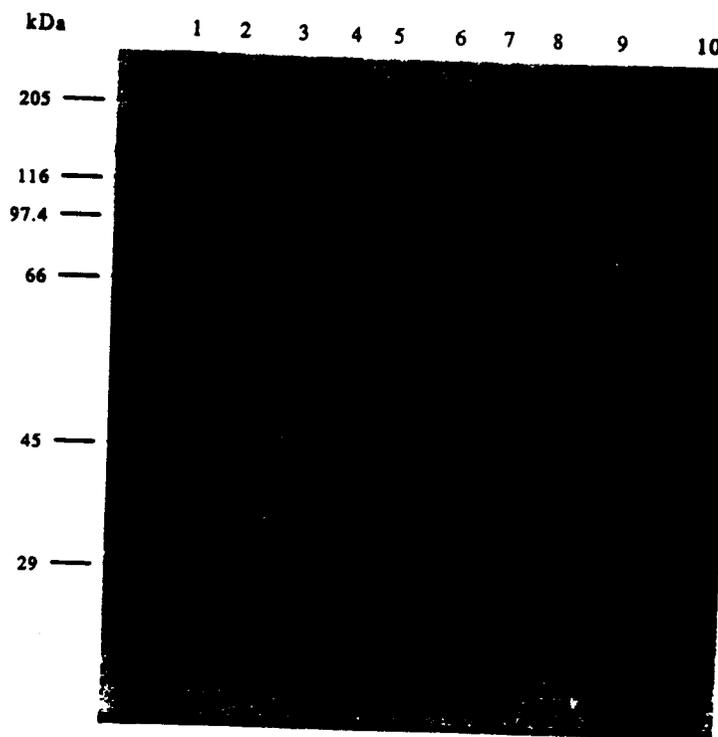


FIGURE 2. *In vitro* translation of *M. sexta* day 6 fifth instar larval mRNA hybrid-selected by clone 10. Single-stranded DNA corresponding to the two orientations of the insert DNA of clone 10 in M13 was immobilized on diazotized aminothiophenol paper and used to hybrid-select mRNA as described in Maniatis *et al.* (1982). The bound RNA was eluted and translated in a reticulocyte lysate using [<sup>35</sup>S]methionine as the radioactive amino acid. The translation products were analyzed by electrophoresis in an SDS-polyacrylamide gel (9%) followed by autoradiography. The autoradiogram was exposed for 16 h. The RNAs used for translation were: lane 1, total RNA; lanes 2 and 3, clone 10 plus strand hybrid-selected mRNA using 1 and 2 mg of total RNA, respectively; lanes 4 and 5, clone 10 minus strand hybrid-selected RNA using 1 and 2 mg total RNA, respectively; lanes 6, 7, 8 and 10, proteins immunoprecipitated with chitinase IgG from the following: 6, molting fluid; 7, total RNA translation product; 8, clone 10 plus strand hybrid-selected mRNA translation product; 10, clone 10 minus strand hybrid-selected mRNA translation product immunoprecipitated with preimmune serum. Molecular weight markers (in kDa) are shown on the left.

through day 8 fifth instar larvae were probed with <sup>32</sup>P-labeled insert DNA from clone 10. Figure 7 shows that chitinase mRNA was present at relatively high levels in epidermis on day 0, but it disappeared rapidly and was undetected on days 1–4 of fifth instar. Chitinase mRNA reappeared on day 5 and peaked on day 7, after which a sharp decline occurred. In the gut, it was detected primarily on day 6 with lower levels present on days 0, 7 and 8. Thus, tissue- and development-specific expression of chitinase genes occurred in *M. sexta* during the larval-pupal transformation. Chitinase gene expression in epidermis occurred 1 day earlier and was somewhat greater than that in the gut.

#### Hormonal control of expression of chitinase genes

The roles of morphogenetic hormones in the expression of *M. sexta* chitinase genes was examined in epidermal and gut tissues from fifth instar larvae that had been ligated on day 2 between the first and second abdominal segments and injected with 20-hydroxyecdysone at both 48 and 72 h following ligation. Chitinase mRNA had declined to a minimal level by day 2 (Fig. 8, day 0). Slot blot analyses showed that, in both epidermis and gut from control abdomens, chitinase mRNA remained extremely low from 3 to 8 days

following ligation, and no pupal cuticle formation was observed. In 20-HE-injected abdomens, the mRNA levels in epidermis sharply increased 4 days after ligation (the second day after the first hormone administration), reaching a peak level of >10-fold higher than that of solvent-injected abdomens [Fig. 8(A)]. Furthermore, pupal cuticle formation was evident in the hormone-treated abdomens by about day 6. In the gut, chitinase mRNA began to increase 1 day following hormone injection, also reaching a level that was >10-fold higher than that in solvent-injected abdomens [Fig. 8(B)]. No decline in chitinase mRNA was observed in the gut 4 days after 20-HE treatment. The maximal level of chitinase mRNA expression in the epidermis was 10–20-fold greater than that in the gut when expressed on a per  $\mu$ g RNA basis. These data clearly showed that ecdysteroid enhanced chitinase mRNA expression by >10-fold in both epidermal and gut tissues of *M. sexta*, but that the epidermis was more responsive than the gut.

To determine the effect of juvenile hormone on the expression of the *M. sexta* chitinase genes, a juvenile hormone mimic, fenoxycarb, was topically applied to ecdysteroid-treated and untreated fifth instar ligated abdomens. Slot blot analyses showed only a slight increase in chitinase mRNA levels in the epidermis of fenoxycarb-

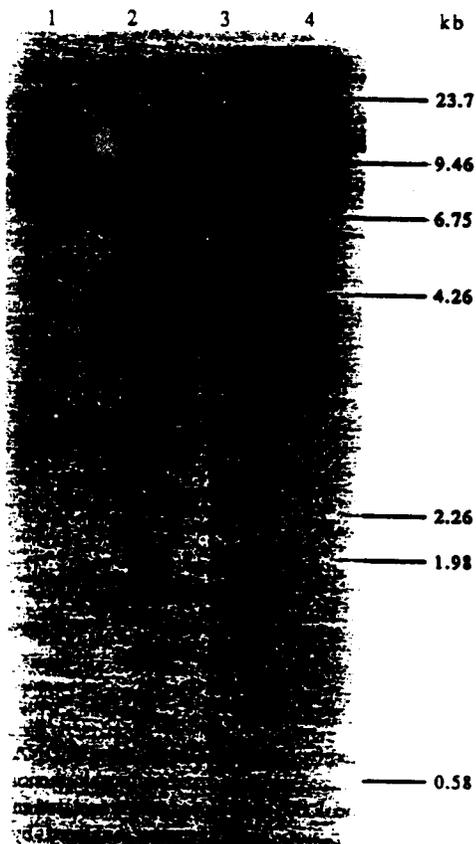


FIGURE 3. Southern blot analysis of *M. sexta* genomic DNA using  $^{32}\text{P}$ -labeled insert DNA from clone 10 as probe. DNA (10  $\mu\text{g}$ ) was digested with restriction enzymes *Sma*I (lane 1), *Hind*III (lane 2), *Eco*RV (lane 3), and *Bam*HI (lane 4). Marker fragments in kilobases (kb) are indicated on the right.

carb-treated abdomens compared to that observed in untreated abdomens, but there was no effect on gut mRNA levels [Fig. 8 (A) and (B)]. When 20-hydroxyecdysone (7.5  $\mu\text{g}/\text{g}$ ) and fenoxycarb (5.4  $\mu\text{g}/\text{g}$ ) were simultaneously administered, chitinase mRNA levels in both epidermis and gut were similar to those observed when juvenile hormone alone was topically applied and 5 to 10 times lower than the levels in abdomens injected with ecdysteroid alone. These data demonstrated that juvenile hormone suppresses the ecdysteroid-induced accumulation of chitinase mRNAs.

## DISCUSSION

Chitinase cDNAs have been isolated from a number of sources including bacteria, yeast, and plants (Broglie *et al.*, 1986; Jones *et al.*, 1986; Kuranda and Robbins, 1991). Although chitinases have been purified from several species of insects, no similar reports have been published on chitinase clones from insects (Kramer and Koga, 1986). In this paper, we describe the isolation and sequence analysis of an apparent full length cDNA clone (clone 201) that encodes a chitinase-like protein from the tobacco hornworm. The protein product of a hybrid-selection experiment using this cDNA reacts with an antibody raised against a chitinase purified from *M.*

*sexta* molting fluid. In addition, the developmental profile of mRNA accumulation is consistent with the expression of chitinolytic enzyme activity *in vivo*. Finally, the amino acid sequence of the encoded protein shows strong sequence similarity to endo- $\beta$ -*N*-acetylglucosaminidases from microbial and plant sources. Therefore, it is very likely that this clone codes for one of the *M. sexta* chitinases.

The amino acid sequence identity between the *M. sexta* chitinase and chitinases from other sources is relatively low overall. The percent identity between *S. plicatus* and *S. cerevisiae* chitinases and the *M. sexta* protein is 20 and 22%, respectively. However, *M. sexta* chitinase contains two regions that are very highly conserved among several microbial and some plant chitinases (see Fig. 5). In particular, several invariant aspartic acid and glutamic acid residues occur in the second conserved region. Because acidic amino acid residues are common in the active sites of polysaccharidases (Henrissat, 1990), it has been postulated that one or more of the acidic residues in this second conserved region participate in catalysis and/or substrate binding.

Another interesting feature of the amino acid sequence of *M. sexta* chitinase is the high frequency of occurrence of serine and threonine residues between positions 396 and 453. Nearly 50% of the amino acids in this region are residues that can potentially act as acceptor sites for *O*-glycosylation. Yeast chitinase also contains a similar serine-threonine rich region (Kuranda and Robbins, 1991). The yeast protein exhibits an electrophoretic mobility corresponding to a molecular mass of about 130 kDa, whereas the primary translation product is much smaller, only *c.* 60 kDa. Apparently extensive glycosylation of the yeast chitinase occurs, and the posttranslationally modified product exhibits an electrophoretic behavior consistent with that of a rather large glycoprotein. Like the yeast enzyme, chitinases in *M. sexta* molting fluid are glycosylated (Koga *et al.*, 1983a). Potential *N*-glycosylation sites in the amino acid sequence occur at positions 85–87, 303–305, 407–409 and 545–547 (Fig. 4).

A discrepancy in molecular mass exists between the size of the hybrid-selected primary translation product of our clone and the protein encoded by the open reading frame of clone 201. The open reading frame of 1662 nucleotides encodes a protein with a calculated molecular mass of 62 kDa, a size identical to one of the three chitinases isolated from *M. sexta* molting fluid (Koga *et al.*, 1983a). However, the major protein in the translation products of mRNA hybrid-selected by the chitinase cDNA clone has an apparent molecular mass of about 75 kDa, a size equal to that of another chitinase present in molting fluid (Koga *et al.*, 1983a). It is unclear why the molecular weight of the primary translation product of this clone is different from that of the predicted molecular weight of the encoded protein. Because the reticulocyte is not known to bring about posttranslational processing, we conclude that this protein has an anomalous electrophoretic migration in

NUCLEIC ACID SEQUENCE OF AND PROTEIN CODED BY CHITINASE CLONE 201

	GAATTCCTCGCCGACACCCGGCTACGTTCAAA	33
ATCGGAGCGACTGGCGCGTGGCTGTCTGGCCCTTAGCGACGGCGGTTCAATCGGACAGCAGAGCGCCGATAGTATGCTACTTCAGCAATTGGGCGGTGTATCGGCGTGGTAGGG		153
M R A T L A T L A V L A L A T A V Q S D S R A R I V C Y F S N W A V Y R P G V G		40
CGGTACGGCATCGAGACATTCAGTGGAGAAGTGTACCCACATCATTTACTCCTTCATTGGCGCTCACTGAGGGCAACAGCGAAGTACTTATCATTGATCCTGAGTGGATGTAGATAAG		273
R Y G I E D I P V E K C T H I I Y S F I G V T E G N S E V L I I D P E L D V D K		80
AATGGTTCCGCACTTACATCGCTTCGGTCTTCGCATCCAGCGTCAAGTTCATGGTAGCGGTGGCGCGCTGGCCTGAAGGCAGTTCGAAGTACTCTCATATGGTTCACAGAGAGGC		393
N G F R N F T S L R S S H P S V K F M V A V G G W A E G S S K Y S H M V A Q K S		120
ACCCGCATGTCTTTTATCAGGACGGTGTGAGTTCCTCAAGAAGTACGACTTCGACGGTCTAGACCTTGATTGGGAGTACCCAGGAGCCGCTGATCGTGGCGCTCTTTTCTGACAAG		513
T R H S F I R S V V S F L K K Y D F D G L D L D W E Y P G A A D R G G S F S D K		160
GACAAATCTTACTTAGTGAAGAGCTCGGAGAGCATTATCAGGGTGGTAAAGGATGGGAAGTACTGCTGCCGTACCACTGGCTAACTTCAGATTAATGGAGGGTTATCATGTG		633
D K F L Y L V Q E L R R A F I R V G K G W E L T A A V P L A N F R L M E G Y H V		200
CCTGAAGTCTGTGAGGAATAGAGCCTATCCACGTAATGTGTCAGCATCTCGTGGTAACTGGCGTGGGTTGCCGATGTGCACTCGCCTTATACAAAGCTCCTCAGCAGCAGTGGGT		753
P E L C Q E L D A I H V M S Y D L R G H W A G F A D V H S P L Y K R P H D Q W A		240
TATGAGAACTTAACGTGAATGATGGTCTCCATCTTTGGGAAGAGAAGGGTGTCCCTCAAACAAGCTGGTGGTGGTATTCATCTACCGTGCATCTTCCACCTATCTGCTGGCAAC		873
Y E K L N V N D G L H L W E E K G C P S N K L V V G I P F Y G R S F T L S A G N		280
AACAACACGGTCTCGGCACCTTCATCAACAAGGAAGCAGGCGCGGTGACCTCGCCATACACCAATGCTACAGGATTTGGGCTTATTGAGATCTGTACAGAAGTAGACAAGGAT		993
N N Y G L G T F I N K E A G G G D P A P Y T A T N A T G F W A Y Y E I C T E V D K D		320
GACTCGGCTGGACGAAGAATGGGACGAGCAAGGCAAGTCCCTATCCCTACAAGGGCACCCAGTGGGTGGATACGAAGACCCCTCGCAGCGTGGAGATCAAGATGAATGGATTA		1113
D S G W T K K W D E Q G K C P Y A Y K G T Q W V G Y E D P R S V E I K M N W I K		360
CAGAAGGATACCTTGGAGCCATGACTTGGGCTATCGACATGGATGACTTCCAAGGACTGTGGAGAGAAGAACCCATTGATCAAGATCTTCATAAGCAGATGAGCTTACACAGTG		1233
Q K G Y L G A M T W A I D M D D F Q G L C G E K N P L I K I L H K H M S S Y T V		400
CGCCTCTCATACAGAGAACCACCCGACTCCTGAATGGGCGGTCCACCGTCAACCCCTTCGGATCCTCAGAAGGAGATCCGATCCCTACCACCACCCAGCTAAGCAGCTTCT		1353
P P P H T E N T T P T P E W A R P P S T P S D P S E G D P I P T T T T A K P A S		440
ACCACCAACGACCGTGAAGACTACTACCACTACCACAGCAAAACCCTCAGAGCGTATTGATGAAGAGAATGATTAATGTGAGGCCTGAACCAAAACCCGAACCTCAACCAGAG		1473
T T K T T V K T T T T T A K P P Q S V I D E E N D I N V R P E P K P E P Q P E		480
CCTGAAGTGAAGTCCCTACTGAAATGAAGTGGTAGCGAATCTGCAACTCAGACCAAGTATATACCCGATAAGAAACACTGTGATAAGTACTGGCGTGGTCAATGGG		1593
P E V E V P P T E N E V D G S E I C N S D Q D Y I P D K K H C D K Y W R C V N G		520
GAAGCAATGCAAGTCTCTTGTCAACACGGAAACGGTATCAATGTGGAAGTGAAGTGTGACTGGCCTAGCAATGCAACACCGTCCGGAATGTCAACAACCTAAAACACTGTTTTATTTC		1713
E A M Q F S C Q H G T V F H V E L N V C D W P S N A T R R E C Q Q P < 554		
AGGAAGTCAATGATACTCAAATTCGCTCAAATGTCTGATTTGATGGTCTGTACACGTTGAAAGTGTCAATTTGCTATCATAAAGAATTCGATTAATCAGATTCATGGAAGCGT		1833
TAAGATATAGTAATAAGTGTGAATATTGTCGATTTTGTGTTAGTTGCAACATAATACGCCAATGTTTTCTTAACTATGTAAGGCTTGATTTTATTTTATTTTTCATACATAAG		1953
TTACTATTTAAGCAAATGAGTCTCTCGCGACTATAATTGTTCAATACTAATAGGTTGATTTTCCATCCAGTGGTATTTACCGCCTCGAGTTTTTTTTAAGACTGCCATTTTTT		2073
ATATTGTTAAGACAAAATTTTTATTTAAATAGTATAGAATAAATTTGCTCACTTTAGAAAATAGCGAATAAGATTTTCATACCTACCGAAATTTATGATGTCGAATGTGTC		2193
TGTTTTTTTTGTAGAATTACGTTGTATTTCGCTCTGTTCAAAAATCATTAGCAAACTCACGGAGCAAAAATTTCTATTTATTTCTTGGATAAATTTGTTTCGAGTCGGAAGCCA		2313
ATTAGCTGGCTCTGGCTCTGGGAATTTAAATGAATTTTCTCGGCACCTGTGGAAGTGGTCCCGCTACTCTTTAGCTAATTTATTTATTTTATAATATAAGTAAATAATTA		2433
TGATTAATAATTCGGAATTC	2452	

FIGURE 4. Nucleotide and predicted amino acid sequences of *M. sexta* chitinase clone 201.

polyacrylamide gels. The possibility that clone 201 is missing some N-terminal sequence appears unlikely because when the 1.8 kb EcoRI fragment from this clone was inserted into a baculovirus vector and expressed in insect cells, two protein bands of c. 75–80 kDa were produced, which were identical in size to molting fluid chitinases. These proteins reacted with the chitinase antibody and exhibited chitinase activity (Gopalakrishnan *et al.*, unpubl. data). Furthermore, these proteins were secreted into the medium, indicating the presence of a leader peptide in the encoded protein. The presence of a hydrophobic N-terminal region with properties characteristic of leader peptides lends additional support to the notion that clone 201 contains all of the chitinase coding region.

Clone 201 with an insert of 2452 nucleotides appears to be a nearly full-length clone, because the transcript

detected by this clone is about 2600 nucleotides long (Fig. 1). The size of the transcript is consistent with the presence of a poly(A) + tail of 100–200 nucleotides on most eukaryotic mRNAs. We have determined the 5'-terminal sequences of at least five different chitinase clones and found that they all start at or nearly at the same position, i.e. 33 bp upstream from ATG. Furthermore, the putative translation initiation codon at positions 34–36 (Fig. 4) is followed by a typical hydrophobic leader peptide sequence that is expected for a secretory protein. This leader peptide suggests synthesis of chitinase on membrane-bound ribosomes. Determination of the N- and C-terminal sequences of the *M. sexta* chitinases will be needed to confirm that the deduced translation initiation site in clone 201 is indeed correct.

Immunoblot analysis of extracts of *M. sexta* and *Bombyx mori* epidermal tissues suggested that chitinase-

	REGION I	REGION II (ACTIVE SITE)
<i>Manduca sexta</i>	(97) K F M V A V G G W A E G S S K	(136) Y D F D G L D L D W E Y P
<i>Cucumis sativa</i>	(98) K V L L S I G G G A G S Y S L	(142) A V L D G V D F D I E S G
<i>Saccharomyces cerevisiae</i>	(102) K V L L S L G G A S G S Y L F	(147) A V V D G F D F D I E N N
<i>Serratia marcescens</i> Chitinase A	(267) K I L P S I G G W T L S D P F	(305) K F F D G V D I D W E F P
<i>Serratia marcescens</i> Chitinase B	(89) R I M F S I G G W Y Y S N D L	(132) Y G F D G V D I D W E Y P
<i>Streptomyces plicatus</i>	(335) K I L Y S F G G W T W S G G F	(374) D V F D G I D L D W E Y P
<i>Streptomyces plicatus</i> Endo H	(127) K V L L S V L G N H Q G A G F	(164) Y G L D G V D F D D E Y A
<i>Vibrio parahemolyticus</i>	(266) K I P S I G G W T L S D P F	(307) D G V D I D W E F P
<i>Streptomyces erythraeus</i>	(72) D V I P S I G G Y S G S K L G	(106) Y G L K A I D V D I E A T
<i>Bacillus circulans</i> Chitinase A1	(156) K T I I S V G G W T W S N R F	(194) Y N F D G V D L D W E Y P
<i>Bacillus circulans</i> Chitinase D	(258) K V L I S M G G A N G R I E L	(293) Y G F N G L D I D L E G S
<i>Arabidopsis thaliana</i>	(102) K V M L S L G G G I G N Y S I	(146) A V L D G I D F N I E L G
	* * *	* * *

FIGURE 5. Comparison of conserved regions in amino acid sequences of chitinases. The chitinase sequences listed are *M. sexta* (this work), *Cucumis sativa* (Metraux *et al.*, 1989), *Saccharomyces cerevisiae* (Kuranda and Robbins, 1989), *Serratia marcescens* A (Jones *et al.*, 1986), *Serratia marcescens* B (Harpster and Dunsmuir, 1989), *Streptomyces plicatus* (Kuranda and Robbins, 1989), *Streptomyces plicatus* endo H (Robbins *et al.*, 1984), *Vibrio parahemolyticus* (Kuranda and Robbins, 1989), *Streptomyces erythraeus* (Kamei *et al.*, 1989), *Bacillus circulans* A1 (Watanabe *et al.*, 1992), *B. circulans* D (Watanabe *et al.*, 1992) and *Arabidopsis thaliana* (Samac *et al.*, 1990). Numbers in parentheses list position in the amino acid sequence.

related proteins with an apparent molecular mass > 100 kDa are synthesized by the epidermis and secreted into the molting fluid (Koga *et al.*, 1989, 1992). The relationship between the larger immunoreactive proteins previously observed and those encoded by our clone 201 is unclear. It is possible that the former are precursor or glycosylated forms of the mature protein. The sequences of the chitinase cDNA clones from yeast (Robbins *et al.*, 1984) and *M. sexta* (this study) do not support the notion of larger precursor forms of chitinases. Mature chitinases significantly larger than the primary translation products have also been observed in yeast (Kuranda and Robbins, 1991).

Enzymes that hydrolyze  $\beta$ -1,4-*N*-acetylglucosamine linkages have been classified into three families of proteins (Henrissat, 1990). One of these includes the basic plant chitinases; another, the exo-cleaving  $\beta$ -*N*-acetylglucosaminidases; and the third family consists of bacterial, yeast, and cucumber chitinases. The *M. sexta* chitinase studied here appears to belong to the last family because it is similar in sequence to it and not the other two families. The insect chitinase clearly differs from the basic plant chitinases, which lack the conserved regions shown in Fig. 5. Furthermore, the insect and microbial chitinases are devoid of a cysteine-rich, chitin-binding hevein-like domain found in the class I plant chitinases (Shinshi *et al.*, 1990; Chrispeels and Raikhel, 1991). The protein encoded by *M. sexta* clone 201 has a structure that is made up in part of a signal peptide

M E F G

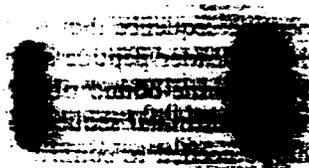


FIGURE 6. Slot blot analysis of *M. sexta* chitinase mRNAs in 5  $\mu$ g total RNA isolated from muscle (M), epidermis (E), fat body (F), and whole gut (G) of on day 6 of fifth instar larvae.

region, chitinase catalytic regions, and a threonine/serine-rich region. It lacks the cysteine-rich chitin-binding region.

Southern blot analyses revealed the presence of one or two chitinase genes in the *Manduca* genome. We have identified only one expressed chitinase gene even after sequencing several chitinase cDNA clones. It is unclear whether only one chitinase gene is functional during the larval-pupal transformation, and others are expressed at different stages of development, or whether the other chitinase genes are pseudogenes. Based on the number of bands in Southern blots seen with the smallest probe and partial DNA sequence of one chitinase genomic clone (Choi *et al.*, unpubl. data), we estimate that there are two chitinase genes in the *M. sexta* genome.

A particularly striking feature of the present study is the stimulation of chitinase gene expression and accumulation of transcripts in epidermal and gut tissues caused by an ecdysteroid. Furthermore, the increase in chitinase mRNA appears to coincide with an increase in the titer of ecdysteroid *in vivo* (Bollenbacher *et al.*, 1975; Riddiford, 1985, 1987). This stimulation represents one of the early events that can be related causally to the hormonal control of insect molting. Accumulation of chitinase mRNA occurs during the middle to late stages of the fifth instar and probably reflects either a phase of rapid transcription of the gene and/or hormone-mediated increase in the stability of the transcripts.

Ecdysteroid and/or juvenoid treatment of ligated *M. sexta* larval abdomens was shown previously to affect the expression of chitinolytic enzyme activity *in vivo* (Fukamizo and Kramer, 1987). Kimura (1973) used an ecdysteroid to induce chitinolytic enzyme activity in larval abdomens of *B. mori*, and Spindler-Barth *et al.* (1986) used a juvenoid to suppress activity in *Ephesia cautella*. Using our chitinase cDNA clone as a hybridization probe, we found that ecdysteroid treatment promoted a 10-fold increase in the level of chitinase mRNAs and that co-administration of a juvenoid with the ecdysteroid prevented this increase. Although we cannot rule

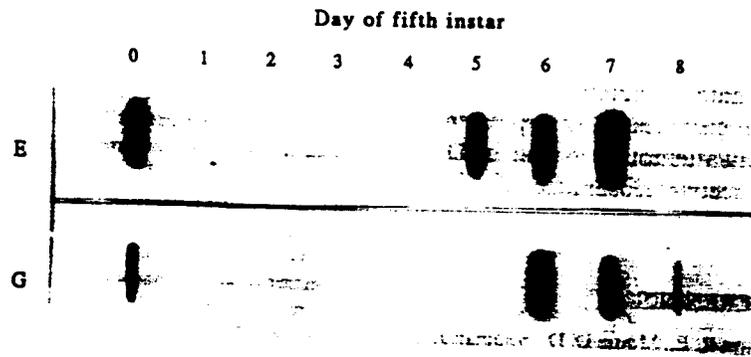


FIGURE 7. Slot blot analysis of *M. sexta* chitinase mRNAs in 2.5  $\mu$ g of total RNA from day 0 through day 8 fifth instar larvae of epidermis (E) and gut (G). Numbers indicate day of fifth instar.

out the possibility that this effect was due to changes in the rates of RNA turnover or processing, the increase in chitinase mRNA levels most likely represents an enhanced level of gene transcription caused by ecdysteroid.

Fukamizo and Kramer (1985a, b, 1987) have proposed that degradation of cuticular chitin is synergistically enhanced by the simultaneous action of chitinase and  $\beta$ -*N*-acetylglucosaminidase. It will be interesting to

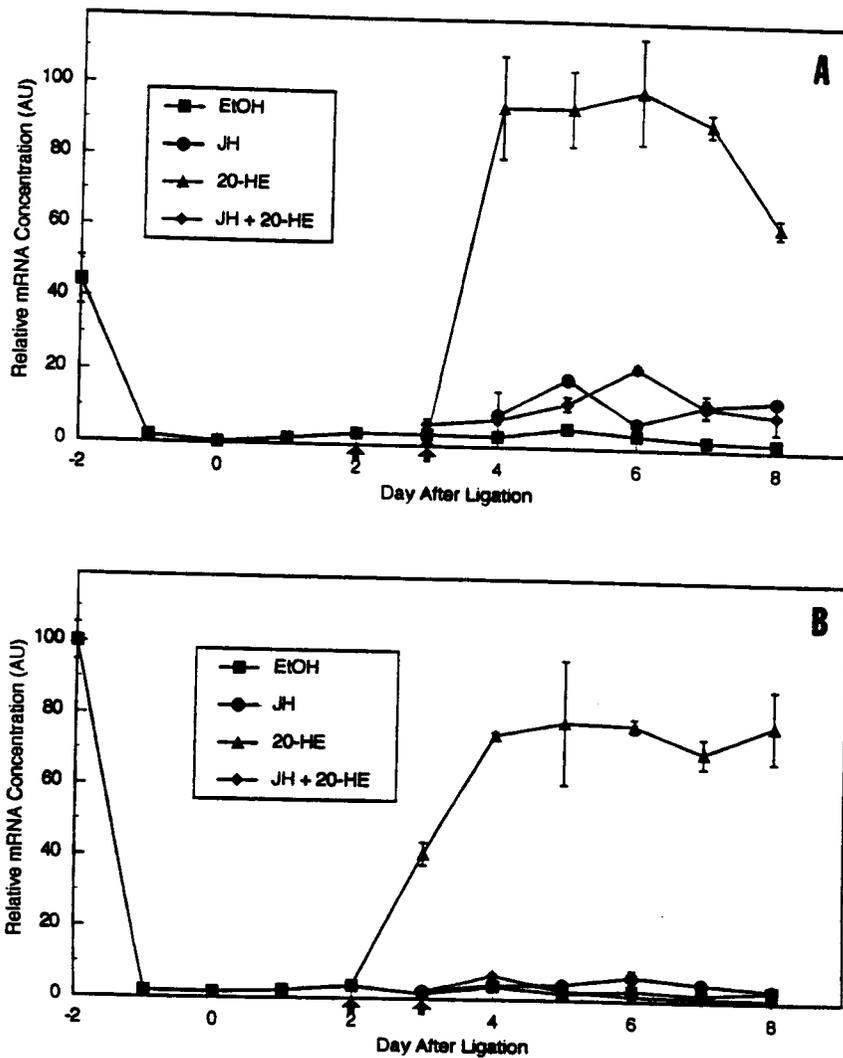


FIGURE 8. Effect of 20-hydroxyecdysone (20-HE) and a juvenile hormone mimic (fenoxycarb) on chitinase RNA transcripts in epidermis (A) and gut (B) of *M. sexta* fifth instar larvae as determined by slot blot hybridization. Six or more larvae were pooled for each mRNA preparation. Day 2 fifth instar larvae were ligated prior to hormone administration (day 0). Days 2 and 1 refer to days before ligation and correspond to days 0 and 1 of the fifth instar, respectively. The arrows indicate the days of hormone treatment. Mean  $\pm$  0.5 range ( $n = 2$ ). Solvent-treated (EtOH) abdomens served as control. Values are expressed as percent of maximum reached in 20-HE-treated larvae.

monitor the accumulation of  $\beta$ -*N*-acetylglucosaminidase transcripts in relation to that of chitinase mRNAs and to investigate the coordinated regulation of mRNAs coding for molting fluid enzymes by morphogenetic hormones. We are currently attempting to isolate cDNA clones for *M. sexta*  $\beta$ -*N* acetylglucosaminidases.

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