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Identification and Preliminary Characterization of a Chitinase Gene in the *Autographa californica* Nuclear Polyhedrosis Virus Genome

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A functional chitinase gene (*chiA*) has been identified in the genome of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV). It is expressed in the late phase of virus replication in insect cells. High levels of both endo- and exochitinase activity were detected by 12 hr p.i. and remained stable throughout infection. An AcMNPV chiA protein-specific antibody was prepared using recombinant material prepared in bacteria. This was used to demonstrate that a product of approximately 58 kDa was synthesised in virus-infected cells. Immunofluorescence analysis of virus-infected cells showed that most chitinase was located in the cytoplasm. Primer extension analysis of mRNA from AcMNPV-infected cells confirmed that transcription initiated from a baculovirus late start site (TAAG), 14 nucleotides upstream from the putative translation initiation codon. The predicted protein sequence of the AcMNPV *chiA* shares extensive sequence similarity with chitinases from bacteria and, in particular, the *Serratia marcescens* chitinase A (60.5% identical residues). Phylogenetic analyses indicate that AcMNPV, or an ancestral baculovirus, acquired the chitinase gene from a bacterium via horizontal gene transfer.

INTRODUCTION

The complete sequence of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) has been published recently (Ayres *et al.,* 1994). The double-stranded, circular DNA genome comprises 133,894 bp with at least 154 nonoverlapping, methionine-initiated potential open reading frames of 150 nucleotides or more. This widely studied member of the *Baculoviridae* serves as the prototype virus for the family. The availability of the sequence data has permitted a detailed analysis of the genetic composition of this insect-specific virus. One of the genes identified in the AcMNPV genome appeared to encode a chitinase. In this paper we describe the detection of a functional chitinase in virus-infected cells. We also make some observations on the relatedness of this gene with chitinases from other species and speculate on the possible origin of the virus-encoded chitinase, in view of its close similarity with the *Serratia marcescens* chitinase A gene (*chiA*) (Jones *et al.,* 1986).

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METHODS

Cells and virus

The AcMNPV C6 clone (Possee, 1986; Possee *et al.,* 1991; Ayres *et al.,* 1994) was propagated in *Spodoptera frugiperda* cells (IPLB-SF21; Vaughn *et al.,* 1977), maintained in TC100/10% foetal calf serum, as described previously (King and Possee, 1992).

DNA sequencing and primer extension analyses of mRNA

Sequencing reactions were performed using a Sequenase Version 2.0 sequencing kit, as described by Ayres *et al.* (1994). Primer extension of mRNA from uninfected or AcMNPV-infected *S. frugiperda* cells was carried out as reported previously (Possee *et al.,* 1991) using a synthetic oligonucleotide (P-CHIT, 5′CGGTGTCTGCGACTTTGACC-3′; 354–373 bp relative to the translation initiation codon). The synthetic oligonucleotides employed in these methods were prepared using an Applied Biosystems Instruments synthesizer (Model 380B; Warrington, UK).

Analyses of chitinase genes

Chitinase sequences similar to the AcMNPV *chiA* were obtained by searching protein databases using the FastA

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program. Matches with extensive similarity along their entire sequences were further investigated for their significance using a Monte Carlo routine in MULTALIGN (Barton and Sternberg, 1987). Sequences showing more than 1 SD above mean random matches were further selected by Dot–Plot analysis for determining the positions of the conserved core domains relative to the AcMNPV chitinase. After removing domains which were not conserved with the AcMNPV chitinase, the various chitinases were analysed using multiple alignment programmes: Gene Works 2.2 (Intelligenetics), Multalin (Corpet, 1988), and MULTALIGN. The outputs were used in phylogenetic estimations and for the determination of mutational regimes and compositional biases.

The phylogeny of the AcMNPV chitinase gene was reconstructed, using protein data sets, by parsimony with PAUP 3.1 (Swofford, 1993) and MacClade 2.2 (Maddison and Maddison, 1992), and distance methods: Fitch-Margoliash and neighbour-joining methods in PHYLIP 3.5 (Felsenstein, 1993). For the determination of the likelihood of alternative topologies and, therefore, alternative hypotheses of the origin of the AcMNPV chitinase gene, a data set including sequences showing the highest conservation at the DNA level to the AcMNPV chitinase was analysed using the maximum likelihood method (Felsenstein, 1993). In addition, the same data set was used for an evolutionary invariant analysis (Swofford, 1993) to investigate the level of support for the origin of the AcMNPV chitinase gene.

The G+C compositional bias of three chitinase genes from different species was determined for their entire genes and compared with that from the fourfold degenerate sites using the methods of Wu and Li (1985). Fourfold degenerative sites were investigated since they are a better indicator of genomic compositional biases, independent of protein coding contraints (Li and Graur, 1991; Sharp, 1990). The evolutionary regime of the AcMNPV chitinase gene was investigated using the DNA sequence alignment for the estimation of the rates of synonymous substitutions (d_s) which do not change amino acids and nonsynonymous substitutions (d_n) which change amino acids per synonymous and nonsynonymous sites, respectively, using the proportional distance method in MEGA (Kumaru *et al.*, 1993). Using d_s and d_n values we tested for rate variation along different lineages using the relative-rate test (Sarich and Wilson, 1973).

Analysis of chitinase activity in insect cells

Mock- and virus-infected (10 PFU/cell) cells were harvested into the medium at various times after infection, pelleted, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS. The cell-free medium from virus-infected cultures was also retained. The levels of chitinase in each sample were determined using the microtitre assay of McCreath and Gooday (1992). The method is based on the detection of fluorescence released by one of four fluorigenic substrates which are 4-methylumbelliferyl glycosides of *N*-acetylglucosamine oligosaccharides [4MU-$(GlcNAc)_{1-4}$], referred to as substrates 1–4. The fluorescent aglycone is released in the presence of the following enzyme activities: *N*-acetylglucosaminidase (substrate 1, 4MU-GlcNAc), exochitinase [substrate 2, 4MU-$(GlcNAc)_2$], endochitinase [substrates 3, 4MU-$(GlcNAc)_3$, and 4, 4MU-$(GlcNAc)_4$] (cf. Robbins *et al.*, 1988).

Production of chitinase-specific antiserum

The pGEX bacterial expression vector system (Smith and Johnson, 1988) was employed to produce a fusion protein comprising glutathione *S*-transferase (GST) and a fragment of the chitinase protein. An internal portion of the chitinase coding sequence (306–965 bp relative to the translational start site) was amplified by PCR, using the oligonucleotide primers F-CHIT (5′-GGGGATCCTGTCAAACTTTGCAATGAGGACGG-3′) and R-CHIT (5′-GGCGAATTCACCGTCCGCATCGCCCAACGA-3′), which added *Bam*HI and *Eco*RI restriction enzyme sites (underlined) to the 5′ and 3′ ends of the reaction product. The fragment was treated with *Bam*HI and *Eco*RI and ligated into *Bam*HI/*Eco*RI-digested pGEX-1 (Pharmacia), producing pGEX-CHIT. Following the protocol of Smith and Johnson (1988), pGEX-CHIT-transformed *Escherichia coli* XL-1 blue cultures were induced to synthesise fusion protein using 0.1 m*M* IPTG and incubated for 18 hr at 37° with shaking. To identify fusion protein synthesis, samples were analysed using SDS–polyacrylamide (10%) gels (Laemmli, 1970), followed by Coomassie blue staining or Western blot analysis (Merryweather *et al.*, 1990) with GST-specific antiserum (1/1000) (Sigma Chemical Co.).

Purification of the GST–chitinase fusion protein using Triton X-100-mediated lysis of bacterial culture and binding to glutathione–agarose beads (Smith and Johnson, 1988) was not possible due to the protein's insolubility under these conditions. However, a unique band of approximately 58 kDa, consistent with the predicted size of the fusion protein, cross-reacted with the anti-GST antiserum in Western blot analysis and was clearly visible in IPTG-induced pGEX-CHIT-transformed cell samples. This band was excised from polyacrylamide gels which had been stained with 0.25 *M* KCl and was soaked in sterile water at 4° for 48 hr to remove the KCl. The strip was then macerated for immunization of guinea pigs. The macerated product from one polyacrylamide gel was mixed with an equal volume of adjuvant for each injection. For the initial injection, Freund's complete adjuvant (Sigma Chemical Co.) was used. Subsequent injections utilized Freund's incomplete adjuvant (Sigma

Chemical Co.). Five injections were administered per animal, at 9- to 12-day intervals. The terminal bleed was taken 9 days following the last injection, and the antiserum was tested in Western blot analysis and indirect immunofluorescence microscopy.

SDS–PAGE and Western blot analysis of mock- and virus-infected cells

Virus-infected and mock-infected cell monolayers were harvested at various times p.i. After pelleting and washing in PBS, the cells were resuspended in sample buffer (Laemmli, 1970). Approximately 10^5 cells were analysed by SDS–PAGE using 12% gels (Laemmli, 1970), followed by Coomassie blue staining or Western blot. For Western blot analysis, anti-chitinase antiserum was diluted 1/10,000 and labelled with alkaline phosphatase-conjugated goat anti-guinea pig antiserum (Sigma Chemical Co.).

Indirect immunofluoresence microscopy

Droplets containing mock- or virus-infected (18 hr p.i.) cells (7×10^4 cells) were placed on Teflon-coated multispot microscope slides and air-dried. After fixing in methanol for 10 min at −20°, a standard two-step immunofluorescence assay was performed. Slides were first incubated with anti-chitinase antiserum (1/500 in PBS, 10 μl per spot) in a humidified environment for 30 min at 37°. They were then washed exhaustively in PBS and incubated with FITC-conjugated goat anti-guinea pig IgG polyclonal antiserum (Sigma Chemical Co.) (1/1000 in PBS, 10 μl per spot) in a humidifed environment at 37° for 60 min. Slides were then washed in PBS, dried, and mounted in 1,4-diazobicyclo-(2,2,2)-octane-based antifading mountant (Johnson *et al.*, 1982). Slides were examined immediately using an Olympus BHS fluorescence microscope with a 100-W mercury vapour bulb, at wavelengths of 405 and 435 nm. An exciter filter (BP-490), supplementary exciter filter (EY-455), and additional O-515 and G-520 filters were used. Kodak 400 ASA Tri-X film was used for photography.

RESULTS

The AcMNPV genome contains a chitinase gene

In a speculative search for chitinase activity in cultured insect cells, analysis of AcMNPV-infected *S. frugiperda* cells, several days after infection, provided the first evidence that a functional chitinase was induced (G. Gooday, unpublished data). This prompted a search of the complete sequence of AcMNPV (Ayres *et al.*, 1994) for coding regions with similarity to known conserved peptide domains representing the predicted active site of a range of chitinases (Kuranda and Robbins, 1991), using the TFasta programme. This analysis identified a similar protein sequence encoded by a region within a 1653-nucleotide open reading frame (ORF) (105,282–106,935), antisense to the polyhedrin gene. This ORF could encode a protein with a predicted M_r 60.9 kDa.

Analysis of chitinase activity in mock- and virus-infected cells

Our preliminary studies had identified a functional chitinase in AcMNPV-infected cells. We extended this observation by examining chitinase production throughout the course of virus infection. Monolayers of *S. frugiperda* cells were infected with AcMNPV and harvested at intervals of 6 hr. Cell extracts and tissue culture medium were analysed using the microtitre assay of McCreath and Gooday (1992) (Fig. 1). The assay was tailored to detect endo- and exochitinase activity in samples by selective use of different substrates (see Methods). Very low levels of chitinase activity were detected in uninfected cells (Fig. 1a), contrasting with high endo- and exochitinase activity in AcMNPV-infected cells (Fig. 1b). Chitinase activity was first detected between 6 and 12 hr p.i. This reached a maximum level at 12 hr p.i., which was maintained until at least 36 hr p.i. *N*-Acetylglucosaminidase activity was low in both virus-infected and uninfected cells. Only low levels of chitinase (<10% of total activity) were detected in the cell culture medium of virus-infected cells. In contrast, in cultures of uninfected cells, approximately 90% of the total activity was in the medium (data not shown). To identify the initiation of chitinase synthesis in AcMNPV-infected cells more accurately, samples were taken at hourly intervals between 6 and 12 hr p.i. and again at 24 hr p.i., before determining endochitinase activity using substrate 3 (Fig. 1c). Chitinase activity was detected at 8 hr p.i. in AcMNPV-infected cells and reached a peak at 12 hr p.i.

Detection of the chitinase protein in virus-infected cells

An antiserum was raised against a fusion protein comprising GST and a portion of the chitinase protein. The oligonucleotide primers (F-CHIT and R-CHIT) used for PCR amplification of the chitinase gene are described under Methods. The specificity of this antiserum was assessed by Western blot analysis of extracts from virus-infected cells harvested between 3 and 18 hr p.i. and uninfected *S. frugiperda* cells (Fig. 2). A protein of about 58 kDa was detected only in AcMNPV-infected cells from 10 hr p.i.

The same antiserum was used in indirect immunofluoresence microscopy analysis to locate chitinase within AcMNPV-infected cells (Fig. 3). Mock- or virus-infected cells were examined at 18 hr p.i. A strong fluorescence was detected only in AcMNPV-infected cells (Fig. 3A). Within these cells, cross-reactive antigen appeared to aggregate in cytoplasmic foci, superimposed upon a

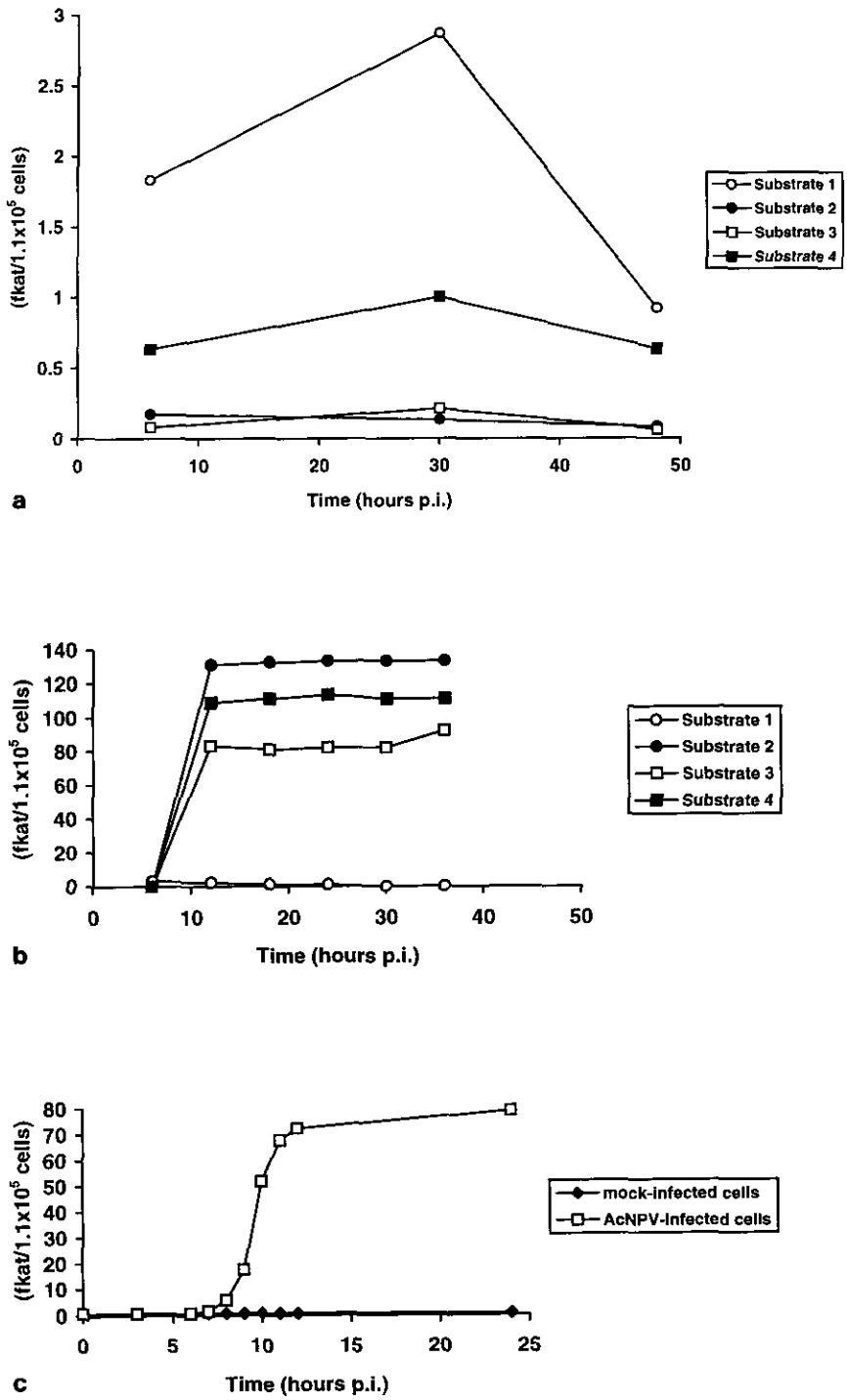

FIG. 1. Induction of chitinase activities in AcMNPV-infected cells. (a) Mock-infected and (b) *S. Frugiperda* cells were inoculated with AcMNPV (10 PFU/cell). Cells were harvested at the times indicated and assessed for *N*-acetylglucosaminidase (○), exo- (●), and endochitinase (□, ■) activites in the microtitre assay, with the substrates 4MU-GlcNAc, 4MU-$(GlcNAc)_2$, 4MU-$(GlcNAc)_3$, and 4MU-$(GlcNAc)_4$, respectively. (c) Endochitinase activity (with substrate 3) detected in mock- (◆) and AcMNPV-infected (□) cells between 0 and 24 hr p.i. Activities are expressed in femtokatals (fkat/1.1 × 10^5 cells). A katal represents the hydrolysis of a mole of substrate per second. Note differences in the scales between a and b.

lower level of more evenly distributed cytoplasmic fluorescence. No reactivity was detected in the nucleus or on the cytoplasmic membrane. Minimal cross-reactivity was found in uninfected cells (Fig. 3B).

Transcription of the AcMNPV chitinase gene

The observations on protein production in virus-infected cells suggested that the chitinase gene was tran-

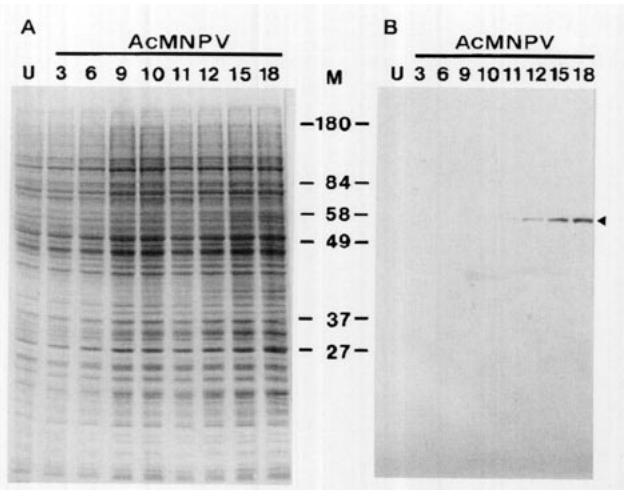

FIG. 2. Immunoblot analysis of chitinase in AcMNPV-infected cells. *S. frugiperda* cell monolayers (10^6 cells) were mock-infected or inoculated with AcMNPV (10 PFU/cell). Virus-infected cells were harvested between 3 and 18 hr p.i. and 10^5 cells were fractionated in duplicate 12% polyacrylamide gels. (A) Coomassie blue-stained gel. (B) Immunoblot analysis using anti-chitinase antiserum (1/10,000) for primary detection and alkaline phosphatase-conjugated goat anti-guinea pig IgG polyclonal antiserum (1/1000) for the secondary reaction. AcMNPV, AcMNPV-infected cells; U, mock-infected cells. the position of the chitinase band in b is indicated with an arrow. Molecular size markers (M; kDa) are shown.

scribed from a baculovirus late gene promoter. This was confirmed by performing primer extension analysis on mRNA extracted from mock- or virus-infected cells at 12 hr p.i. (Fig. 4). A product of 386 nucleotides was derived from AcMNPV-infected cell mRNA which was coincident with a typical late baculovirus transcription initiation signal (TAAG) (Rohrmann, 1986), located 14 nucleotides upstream of the translation initiation codon. Primer extension products were absent in the uninfected cell mRNA samples.

Comparison of the AcMNPV chitinase with chitinases from other organisms

The initial TFasta data revealed that a portion of the AcMNPV chitinase sequence was identical with the putative active site region of the *S. marcescens chiA* (Jones *et al.*, 1986). To enable a comparison of the AcMNPV chitinase with other related protein sequences, the entire amino acid sequence was submitted to the National Centre of Biotechnology Information using the BLAST network service (Altschul *et al.*, 1990). The sequences reported by BLAST were extracted from their respective databases and analysed for similarity to the AcMNPV chitinase sequence. The BLAST search for protein sequences similar to the AcMNPV chitinase did not retrieve any plant chitinase sequences. These latter sequences were extracted manually from the SwissProt databank. The 17 sequences were compared with the AcMNPV chitinase using a Monte Carlo method (Table 1). The highest conservation was with two bacterial enzymes: the enterobacterium *S. marcescens chiA* (Jones *et al.*, 1986) (60.5% identity; 56.14 SD above mean) and the marine bacterium *Alteromonas* sp. strain O-7 *chiA* (Tsujibo *et al.*, 1993) (48.36% identity; 49.83 SD above mean). An alignment of the three proteins is shown in Fig. 5a. The sequences of the AcMNPV and *S. marcescens* chiA proteins are identical in a region (Fig. 5a; positions 309–329) encompassing the likely active site of chitinases (Henrissat, 1990; Watanabe *et al.*, 1992). The protein sequences diverge at the N-termini where the secretory signal sequence of the bacterial enzyme is missing from the AcMNPV sequence. Interestingly, the virus chitinase appears to have a eukaryotic signal peptide (Ayres *et al.*, 1994).

The striking conservation of the AcMNPV *chiA* to the *S. marcescens chiA* at the DNA level was confirmed in a Dot–Plot analysis (data not shown), which also indicated that there was no sequence similarity in the flanking regions of both genes. However, the region upstream of the translation initiation site of the three closely related *chiA* genes from *S. marcescens, Alteromonas* sp., and AcMNPV showed a sequence similar to the TAAG late gene promoter of the AcMNPV *chiA* gene in the same position relative to their ATG codons (Fig. 5b).

Attempts to align the complete sequences of the 17 chitinases described in Table 1 were complicated by the considerable variation in length and conservation between them. Previous reports, however, in which only the likely active sites of chitinases were compared (B. Henrissat, personal communication; Watanabe *et al.*, 1992), cumulatively detail conservation between the plant type III chitinases *Arabidopsis thaliana* acidic chitinase (Samac *et al.*, 1990); *Hevea brasiliensis* hevamine, which has lysozyme/chitinase activity (Jekel *et al.*, 1991); *Cucumus sativus* acidic chitinase (Metraux *et al.*, 1989); and chitinases from yeast and bacteria. More recently, however, the crystal structure of the *S. marcescens* chitinase A has been resolved at the 2.3 Å level (Perrakis *et al.*, 1994). The structure of the chitinase A complexed with the substrate N,N″,N‴,N⁗-tetraacetylochitotetraose suggests that the Glu315 might be the catalytic residue in the active site. We aligned the amino acids encompassing the putative active sites of the plant chitinases with the equivalent regions in the chitinases of AcMNPV, bacteria, fungi, and animalia (Fig. 6). The predicted catalytic residue (Glu315) in the *S. marcescens* chitinase A was conserved in all of the other proteins, except the human gp39, which lacks chitinase activity. An aspartate residue at position 311 was conserved in all of the amino acid sequences which were compared.

Phylogenetic reconstruction of the origin of the AcMNPV *chiA* gene

In order to investigate the origin of the AcMNPV *chiA* we constructed a multiple sequence alignment of 11 chi-

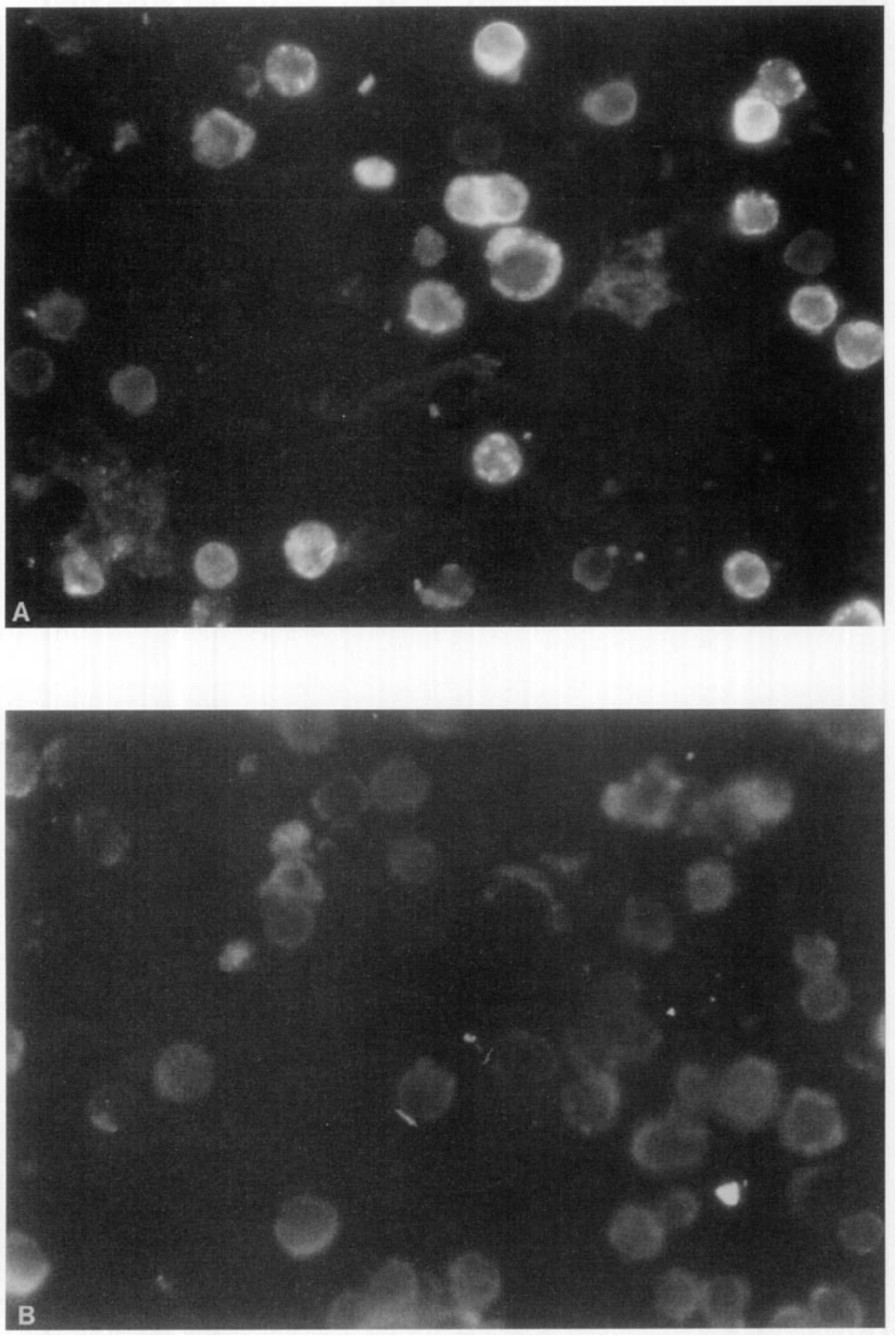

FIG. 3. *Indirect immunofluorescence microscopy of AcMNPV and mock-infected cells. Monolayers of S. frugiperda cells were (A) inoculated with* AcMNPV (10 PFU/cell) or (B) mock-infected with medium. At 18 hr p.i., the cells were harvested, fixed to multispot microscope slides, and incubated *with guinea pig anti-chitinase antiserum before staining with FITC-conjugated goat anti-guinea pig polyclonal antiserum.*

tinase genes showing extensive sequence similarities and included the three animal proteins as an outgroup. Phylogenetic estimations using distance methods (data not shown) agreed with the most parsimonious tree shown in Fig. 7 with respect to the branching order of the AcMNPV *chiA*. Using the animal proteins as an outgroup, the data clearly indicate with 100% bootstrap support that the AcMNPV *chiA* belongs to a bacterial lineage, which confirms the results of the Monte Carlo analysis (Table 1). Moreover, displacing the AcMNPV *chiA* branch

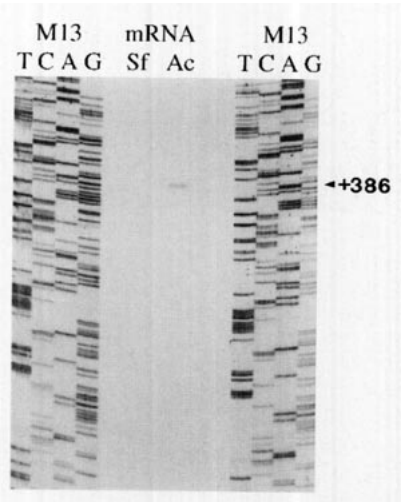

FIG. 4. Primer extension analysis of AcMNPV chitinase mRNA. Polyadenylated mRNA was extracted from total RNA derived from mock-infected (Sf) and AcMNPV-infected (Ac) *S. frugiperda* cells at 12 hr p.i. The oligonucleotide primer used in the primer extension reactions was complimentary to a sequence 373 nucleotides downstream of the translation start site of the chitinase gene. Reaction products were analysed in a 6% polyacrylamide gel, adjacent to an M13 mp18 sequencing ladder to facilitate accurate sizing. The 386-nucleotide chitinase specific product is indicated with an arrow.

from its position caused tree topologies which were significantly worse as determined by statistical tree topology tests for protein data and reduced likelihood values for DNA data (Felsenstein, 1993).

Compositional biases and mutational regimes of the *chiA* genes

A comparison of the G+C utilization in the three closely related chitinase genes is shown in Fig. 8. The G+C content in the fourfold degenerate sites of the AcMNPV *chiA* (C+G, 50.4%; bias, 11.5%; $P \leq 0.05$) shows no significant bias and therefore departs from the expected genome composition of 41% G+C (Ayres *et al.*, 1994). In order to determine if the higher G+C composition was characteristic of that locus in the AcMNPV genome, the two genes flanking *chiA* were also analysed. This indicated that the cathepsin gene (vcath) downstream of *chiA* has 4.1% less G+C than the *chiA* but no significant bias (G+C, 46.3%; bias, 8.8%; $P \geq 0.2$). The ORF 125 has a similar bias (G+C, 40%; bias, 15.6%; $P \leq 0.001$) to that of the AcMNPV genome composition. Moreover, when the three genes were considered, the highest G+C composition observed was for the *chiA* gene (47.7%). Comparing the composition of the AcMNPV to bacterial genes, it can be seen that the *S. marcescens chiA* shows significant bias at fourfold degenerate sites (G+C, 61.6%; bias, 15.6%; $P < 0.1$) and for the entire gene (G+C, 58.6%; bias, 11.5%; $P \leq 0.001$). The *Alteromonas* sp. *chiA*, however, has a bias towards the utilization of A+T at the fourfold degenerate sites (G+C, 41.1%; bias, 11.8%; $P \leq 0.01$). Therefore, considering the AcMNPV genome nucleotide composition, the higher overall G+C composition of the AcMNPV *chiA* gene resembles the *S. marcescens chiA* G+C genomic content and might constitute additional evidence that both genes share a common ancestry.

The DNA sequence alignments between the AcMNPV *chiA* and the two bacterial genes (data not shown) were used to estimate the rates of synonymous substitutions, which do not change amino acids, and nonsynonymous substitutions, which do change amino acid residues. The significantly higher synonymous rates observed in all chitinases ($d = d_s - d_n = 0.4504$; $P < 0.001$) indicate that they are under strong selective pressure at the amino acid level as would be expected for genes under functional constraints. Furthermore, considering the *Alteromonas* sp. *chiA* as an outgroup, the relative rate tests resulted in values near zero for both synonymous ($d_{s_{\Delta K}} = -0.0414$) and nonsynomymous ($d_{n\Delta K} = 0.0242$) substitution classes indicating that after the split from a common ancestor shared with the *Alteromonas* sp. *chiA*, insignificant rate variation was observed among the *S. marcescens* and the AcMNPV *chiA* genes. In summary, these data indicate that the AcMNPV *chiA* gene shares not only high primary sequence conservation with the *S. marcescens chiA* but also preference for high G+C utilization and overall similar evolutionary rates.

DISCUSSION

We have identified a functional chitinase gene in the AcMNPV genome. It appears to be activated in the late phase of virus gene expression in insect cells and utilizes a TAAG motif to initiate transcription at 12 hr p.i. Confirmation that it is an authentic late baculovirus gene will require more extensive analysis of transcription. High levels of chitinase activity were evident in AcMNPV-infected cells late in infection. It remains to be determined whether this reflects high levels of chitinase synthesis, stability, the action of an efficient enzyme, or a combination of these factors. The chitinase extracted from AcMNPV-infected cells hydrolysed all three substrates used in the enzyme assays, demonstrating both endo- and exochitinase activity. The absence of other virus-encoded chitinases, as evidenced by a search of the complete AcMNPV genome sequence (Ayres *et al.*, 1994), suggests that both activities are due to one enzyme. If this proves to be correct, then the production by AcMNPV of a single chitinase with broad substrate range is in contrast with the production by bacteria (Fuchs *et al.*, 1986; Watanabe *et al.*, 1990, 1992), plants (Payne *et al.*, 1990; Sticher *et al.*, 1992), and fungi (St. Leger *et al.*, 1993) of a battery of chitinases with different, narrow enzymatic characteristics. A chitinase-specific antiserum identified a protein of approximately 58 kDa in virus-infected cells using Western blot analysis. Indirect immu-

TABLE 1

Monte Carlo Analysis of AcMNPV *chiA* Gene Compared to Cellular Chitinases

	Size (aa)	Match	% Identities	Random mean	SD of random mean	Score
S. marcescens ChiA	563	6255	60.55	4313.56	34.58	56.14
Alteromonas sp.	820	5855	48.36	4401.50	29.17	49.83
S. plicatus Chi63	610	4818	26.43	4419.88	27.27	14.60
S. lividans	619	4818	24.11	4442.27	28.43	11.31
A. album	423	3875	35.01	3548.35	28.88	11.31
H. sapiens GP39	383	3451	29.46	3252.46	27.34	7.26
K. lactis	1146	4992	27.27	4897.76	21.63	4.36
C. sativus	292	2608	22.60	2557.75	17.83	2.82
B. malayi	504	4108	2.35	4037.90	28.68	2.44
S. plicatus ChiH	313	2747	23.40	2721.80	18.92	1.81
B. circulans ChiD	488	4007	9.92	3963.06	27.29	1.61
M. sexta	554	4283	16.35	4252.03	34.15	0.91
A. thaliana	302	2662	26.25	2643.20	21.74	0.86
B. circulans ChiA1	699	4605	20.36	4592.08	27.76	0.47
H. brasiliensis	273	2430	25.27	2419.30	23.91	0.45
S. erythraea	290	2541	22.49	2532.97	19.16	0.42
S. marcescens ChiB	458	2026	23.76	4021.77	30.79	0.14

Note. The statistical significance of matches of the AcMNPV *chiA* (551 aa) to 17 other chitinase genes found by the FASTA or BLAST searches was estimated by aligning the AcMNPV *chiA* with 100 randomly generated sequences with the same amino acid composition from each cellular chitinase. Sequences shown in decreasing order of standard deviations above random mean (Score) at a 95% confidence limit. Score values higher than 6 are considered as unambiguous indication of homology (Barton and Sternberg, 1987). Sequences used for comparison: *Serratia marcescens* ChiA (Perrakis *et al.*, 1994); D13762:Empro, *Alteromonas* sp.; P11220:SwissProt, *Streptomyces plicatus* Chi63; P36909:SwissProt, *Streptomyces lividans;* P32470:SwissProt, *Aphanocladium album;* P36222:SwissProt, *Homo sapiens* GP39; P09805:SwissProt, *Kluyveromyces lactis;* P17541:SwissProt, *Cucumus sativus;* P29030:SwissProt, *Brugia malayi;* P04067:SwissProt, *Streptomyces plicatus* ChiH; P27050:SwissProt, *Bacillus circulans* ChiD; P36362:SwissProt, *Manduca sexta;* P19172:SwissProt, *Arabidopsis thaliana;* P20533:SwissProt, *Bacillus circulans* ChiA1; P23472:SwissProt, *Hevea brasiliensis;* P14529:SwissProt, *Saccharopolyspora erythraea;* X15208:Empro, *Serratia marcescens* ChiB.

nofluorescence microscopy of AcMNPV-infected cells, using the same chitinase-specific antiserum, revealed a clustering of antigen in cytoplasmic foci. Nuclear localization of the enzyme was not observed.

The protein sequence of the AcMNPV *chiA* was compared with a number of other chitinase sequences from different organisms. The most significant conclusion from this analysis was that the AcMNPV *chiA* is most closely related to bacterial chitinases, in particular, the *S. marcescens chiA* (60.5% identity). Further, the Monte Carlo results clearly indicated that, based on the number of standard deviations above mean from random matches, the probability of both the *S. marcescens* and the AcMNPV sharing a gene with this level of similarity by chance is insignificant. All of the phylogenetic estimations in our analyses support the hypothesis that the AcMNPV *chiA* was transferred horizontally from a bacterial source.

The nucleotide composition of the *chiA* genes provides an additional suggestion of common ancestry of the AcMNPV and *S. marcescens* chitinase genes. The AcMNPV *chiA* has a G+C composition higher than that expected from the AcMNPV genome. The detection of this departure from the genomic bias at the fourfold degenerate sites, which are not under selective pressure dictated by the coding region of the gene, indicate that the A+T pressure of the viral genome has not been able to bring the AcMNPV *chiA* within the AcMNPV composition. Probably the high G+C bias of the *S. marcescens* genome is still detectable in the AcMNPV *chiA*. This could be an indication of common ancestry independent of the phylogenetic reconstructions and the Monte Carlo simulation results. However, we concede that other selective pressures could account for the higher G+C content in the chitinase coding region.

The significant purifying selective pressures detected among *chiA* genes and the almost lack of evolutionary rate variation between the *S. marcescens* and the AcMNPV genes points to the conclusion that the *chiA* gene, when integrated within the AcMNPV genome, had to assume an important functional role before any significant loss of genetic information. In this respect, it is important to notice that the transpositional event possibly involved 5′ flanking regions containing a motif which is one indel or one transition away from the typical baculovirus late gene promoter (TAAG; Rohrmann, 1986). In the *Alteromonas* sp. *chiA*, a TAAG motif postulated to be involved with ribosome binding (Tsujibo *et al.*, 1993) was found at almost the same position in the AcMNPV *chiA* promoter. Therefore, the reconstruction of the functional cistron in the AcMNPV *chiA* would be facilitated by the presence of a late gene promoter-like motif at the mo-

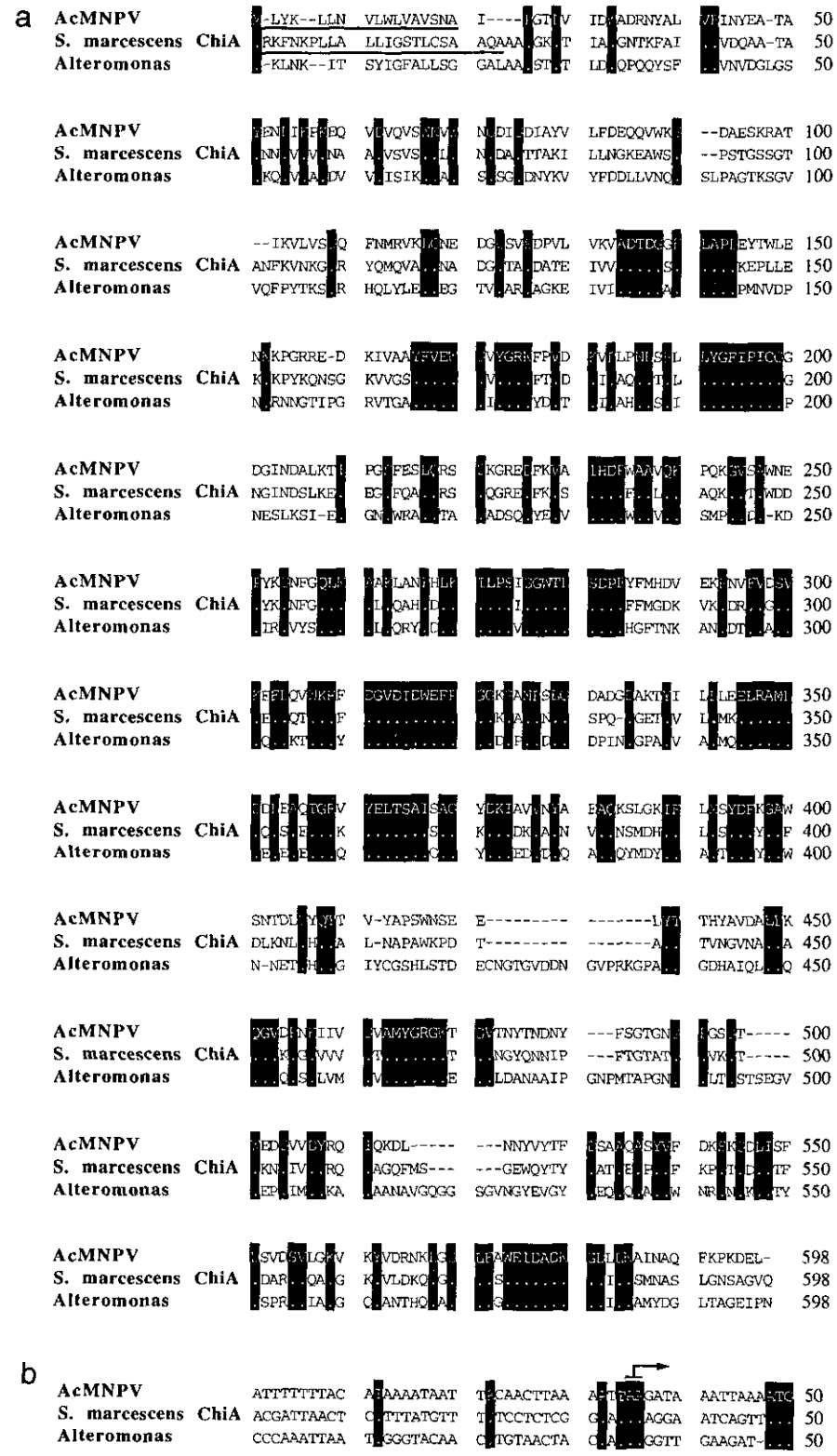

FIG. 5. Comparison of the AcMNPV *S. marcescens* and *Alteromonas* sp. chitinase sequences. (a) Alignment of the amino acid sequences. The putative signal peptide sequence (underlined) for the AcMNPV chitinase comprises the first 17 residues, and for *S. marcescens*, the first 23 residues. (b) Alignment of the 4′ noncoding regions of each gene. The black fills in a and b indicate sequence identity.

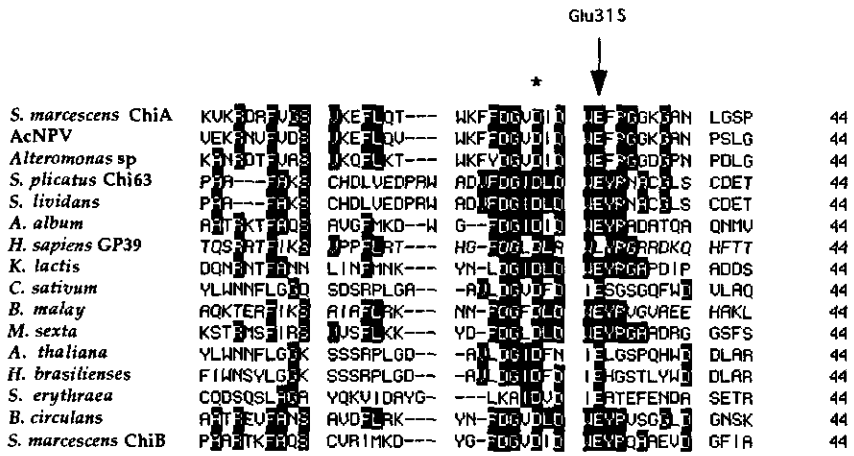

FIG. 6. Alignment of chitinase putative active sites. The amino acids spanning the likely active sites of the chitinases described in Table 1 were aligned. An invariant aspartic acid is indicated with an asterisk. The putative catalytic residue (Glu315) proposed by Perrakis *et al.* (1994) is also indicated. The black fills indicate sequence identity.

ment of transfer or its appearance due to one of three possible single mutational events shortly afterwards.

A functional chitinase gene within the AcMNPV genome might have also resulted from the insertion of the chitinase coding sequence, in frame, within the amino-terminal coding region of a late baculovirus gene. The chitinase gene would, effectively, acquire the promoter necessary for the direction of its own transcription. Some evidence to support this idea is provided by considering the 5′ regions of each gene. The AcMNPV and *S. marces-*

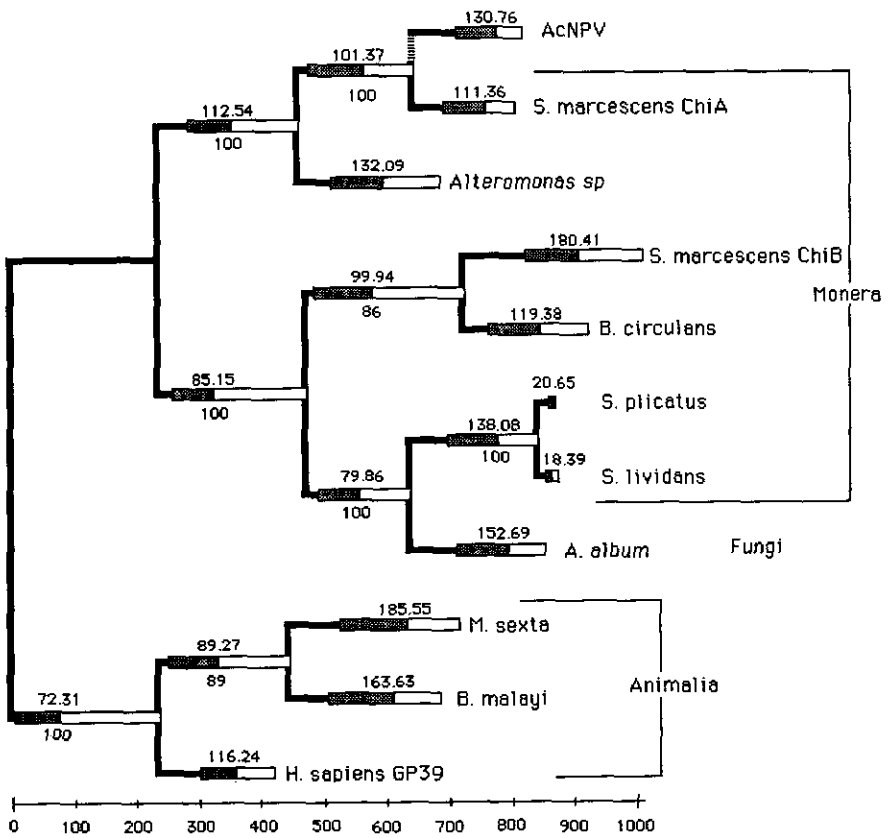

FIG. 7. Phylogeny of the AcMNPV chitinase genes. Eleven cellular chitinases were included in the analysis. Branch lengths and scale are shown proportional to the number of reconstructed changes along branches. The minimum (shaded), average (above branches), and maximum (white) number of changes are shown in the tree branches. Bootstrap values after 100 replications are shown below the branches. The phylogeny determines the orientation of the recombinatorial event since the AcMNPV chitinase gene is nested within a bacterial clade.

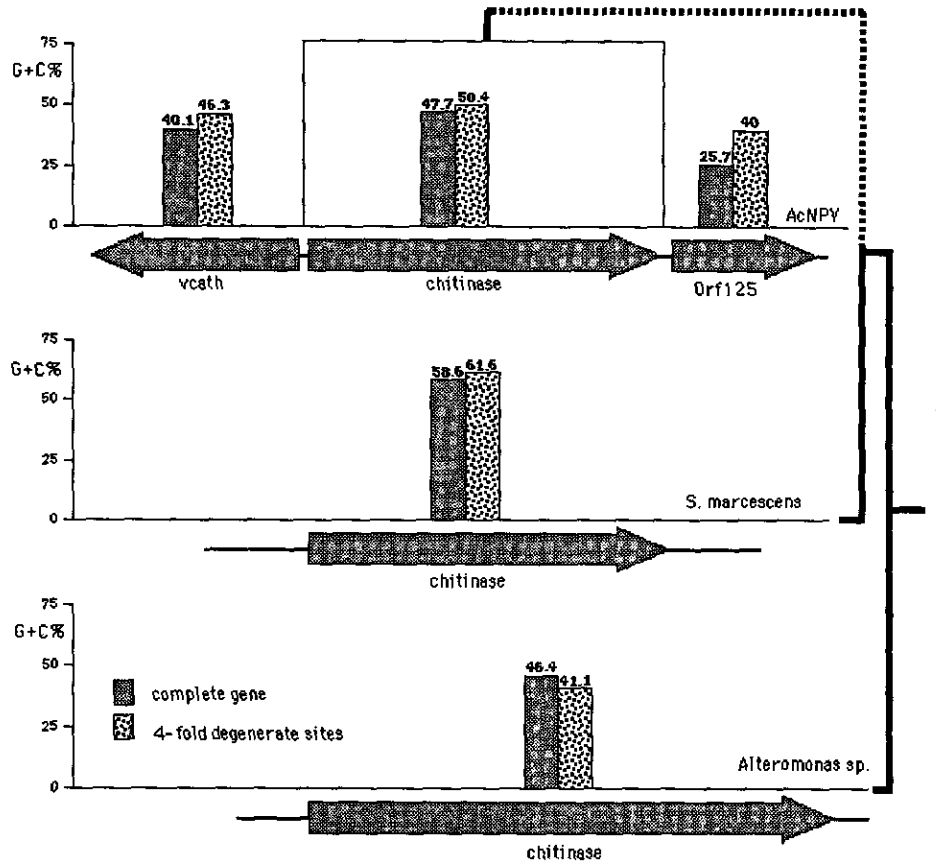

FIG. 8. Graphical comparison of G:C bias of closely related chitinases. The top shows the percentage G:C content of the AcMNPV chitinase and the two flanking genes. The values for the whole coding regons were determined and compared to values obtained from the four-fold degenerate sites. These values are shown above the histogram bars. The process was repeated for the chitinases of *S. marcescens* (middle) and *Alteromonas* sp. (bottom).

cens chitinases diverge at the N-termini, as the 23-amino-acid secretory signal sequence of the bacterial enzyme is not retained in the AcMNPV protein. This may reflect an absence of functional selection pressure in this region due to the redundancy of prokaryotic signal sequences in eukaryotic cells (von Heinje, 1986). The AcMNPV chitinase protein does, however, appear to have a eukaryotic signal sequence at the amino terminus. This does not appear to direct efficient secretion of the protein, since most of the chitinase activity (>90%) is retained within the virus-infected cells. The presence of this signal sequence suggests that AcMNPV might have acquire its chitinase by an in-frame fusion between the bacterial coding region and the original virus gene at this locus. This would have rendered the newly acquired chitinase gene functionally active from the time of its first insertion within the baculovirus genome.

While the idea that the AcMNPV *chiA* was acquired directly form a bacterium is the simplest explanation and supported by the various analyses described in this paper, we cannot exclude alternative proposals concerning its origin. It is feasible that the bacterial gene was integrated within an insect cell genome, prior to its transfer to AcMNPV, or an ancestral baculovirus. Baculoviruses do appear to have acquired an apparent host-derived gene, ecdysteroid UDP-glucosyltransferase (O'Reilly and Miller, 1989; O'Reilly *et al.*, 1992), which may confer an advantage to the virus in prolonging the period of virus production in insect larvae. Analysis of uninfected *S. frugiperda* cells, however, failed to identify significant chitinase activity or the presence of a protein which could cross-react with the chitinase-specific antiserum. Further, the *Manduca sexta* chitinase is very dissimilar to the AcMNPV *chiA*.

If horizontal gene transfer between a bacterium and a baculovirus can account for the presence of the chitinase gene in AcMNPV, the mechanism by which this occurred is unknown. It is worth noting that *S. marcescens* is an enteric insect pathogen and could be present in the host while the baculovirus is replicating. There are precedents for the transfer of DNA between prokaryotes and eukaryotes. The T-DNA segment from the Ti plasmid of certain species of Agrobacterium can be transferred to the genome of plants (a broad range of dicots and monocots)

(Binns and Thomashow, 1988; Zambrynski *et al.*, 1989). Horizontal gene transfer between a prokaryote and eukaryote is also suggested by work reported by Carlson and Chelm (1986). A glutamine synthetase produced by the bacterium *Bradyrhizobium japonicum* was found to have 42.6–46.6% identity with the glutamine synthetase of several plants, but low similarity with another bacterial glutamine synthetase.

Comparison of the AcMNPV chitinase sequence with chitinases from bacteria, yeast, and nematode revealed five major regions of similarity. The positions of these regions are in general agreement with those highlighted by Fuhrman *et al.* (1992), following comparison of *Brugia malayi* chitinase, *S. marcescens* chitinases A and B, *Kluyveromyces lactis* killer toxin α subunit, and *Bacillus circulans* chitinase A. The significance of these regions to the function of chitinases is not known. In the present study, the most striking region of similarity between chitinases encompassed the putative active site. Strong evidence suggesting that Glu315 of the *S. marcescens* chiA protein constitutes the catalytic residue was recently presented by Perrakis *et al.* (1994). This residue was conserved in all of the chitinase sequences compared in this study, including the AcMNPV chiA protein. It is interesting to note that the only protein which lacks this residue was the human gp39 which does not appear to have chitinase activity. It is of note that some plant type III chitinases have both lysozyme and chitinase activities (Bernasconi *et al.*, 1987; Jekel *et al.*, 1991), as the active site of C-type lysozyme comprises an invariant aspartic acid (Asp52) and glutamic acid (Glu35) (Malcolm *et al.*, 1989).

The presence of a functional chitinase gene with high similarity to the chitinase gene of *S. marcescens* (Jones *et al.*, 1986), within the AcMNPV genome, is a surprising result. The remarkable conservation of DNA and protein sequences between the chitinase genes of these two species is suggestive of a transfer of the gene from a bacteria to the virus, albeit via an unknown route.

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