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Antheraea pernyi Nucleopolyhedrovirus *p143* Gene: Nucleotide Sequence and Ineffectiveness in Host Range Expansion of *Autographa californica* Nucleopolyhedrovirus

Ken-ichi Maegawa, Jun Kobayashi and Tetsuro Yoshimura

Faculty of Engineering, Mie University, Tsu, Mie 514-8507, Japan

Abstract **The nucleotide sequences of 3.2- and 4.9-kbp *Pst*I P and H fragments of the *Antheraea pernyi* nucleopolyhedrovirus (AnpeNPV) containing the *p143* gene were determined. A putative DNA helicase of 1212 amino acids was encoded by the gene and exhibited high sequence identities of 58% to 78% with the *p143* gene products of other group I NPVs. Seven conserved helicase motifs, a leucine zipper motif, and two nuclear localization signals, which are conserved among the baculovirus DNA helicases, were also found in the AnpeNPV *p143* gene product. The AnpeNPV *p143* gene introduced in the AcNPV genome were co-expressed with the AcNPV *p143* gene at early phase of infection, but did not enhance either BV production at late phase or recombinant protein production at very late phase in AnPe cells. Thus, the AnpeNPV *p143* gene was not effective in host range expansion of AcNPV to semipermissive AnPe cells, although the result was not conclusive.**
Key words: nucleopolyhedrovirus, *p143*, *Antheraea pernyi*, *Autographa californica*, host range

Introduction

Nucleopolyhedroviruses (NPVs), classified in the family *Baculoviridae*, are large double-stranded DNA viruses that infect arthropods, particularly insects, and are of interest for use as viral insecticides against lepidopteran larvae and as eukaryotic gene expression vectors. In general, NPVs have a restricted host range. Although the mechanisms governing host range restrictions are poorly understood, five different virus genes (the antiapoptotic inhibitors *p35* and *iap*, the DNA helicase homolog *p143*, host cell factor *hcf-1*, and host range factor *hrf-1* genes) that influence the ability of *Autographa californica* NPV (AcNPV) to propagate in nonpermissive and semipermissive insect cell lines have been identified (Miller and Lu, 1997). Deletion of *p35* severely limited the ability of AcNPV to propagate in Sf21 cells but not in Tn368 cells (Clem *et al.*, 1991). Recombinant AcNPV that contain *iap* genes from other baculoviruses in place of *p35* are able to replicate normally in Sf21 cells (Birnbaum *et al.*, 1994; Crook *et al.*, 1993). Replacement of the AcNPV *p143* with the homologous region from the BmNPV *p143* provides AcNPV with capacity to replicate in nonpermissive BmN cells (Kamita and Maeda, 1993; Maeda *et al.*,

1993; Croizer *et al.*, 1994; Argaud *et al.*, 1998). The *hcf-1* is required for AcNPV replication in Tn368 cells and in BTI-TN5B1-4 cells (Lu and Miller, 1995, 1996). The *hrf-1* is required for AcNPV replication in nonpermissive gypsy moth *Lymantria dispar* and *L. dispar*-derived cell line (IPLB-LD652Y) (Thiem *et al.*, 1996; Chen *et al.*, 1998).

We have established a baculovirus expression vector (BEV) system using *Antheraea pernyi* NPV (AnpeNPV) for the high level expression of foreign genes in cultured NISES-AnPe-428 (AnPe) cells derived from *A. pernyi* embryos (Inoue and Hayasaka, 1995) as well as in larvae and diapausing pupae of wild silkmoths such as *A. pernyi and Samia cynthia pryeri* (Wang *et al.*, 2000; Kobayashi *et al.*, 2001). When comparing to other BEV systems such as *Autographa californica* NPV vector with Sf9 and High5 cells and *Bombyx mori* NPV vector with BmN4 cells and *B. mori* larvae, the AnpeNPV-infected diapausing puape of *S. c. pryeri* showed the highest protein production efficiency (Huang *et al.*, 2001; Kobayashi, 2001). In addition, it has been found that the structure of some N-glycans added to the recombinant glycoprotein by AnPe cells is biantenary complex type which is not detected in Sf9 cells but typical in mammalian cells, suggesting that AnPe is well suited for producing pharmaceutical glycoproteins with mammalian-like N-glycans (Nagaya *et al.*, 2002, 2003).

Recently, a comparative genome map of AnpeNPV aligned with the fully sequenced *Orgyia pseudotsugata* multicapsid NPV (OpMNPV) genome (Ahrens *et al.*, 1997) was constructed and the *p143* gene, one of the 5 host range determination genes of AcNPV, was identified in the AnpeNPV genome (Huang *et al.*, 2002). Although both pupae and cell line of *A. pernyi* do not permit productive replication of AcNPV, the introduction of the intact AnpeNPV *p143* gene into the AcNPV genome may expand the host range of AcNPV. The development of such a host-range-expanded AcNPV, which can propagate and produce recombinant proteins in *A. pernyi* diapausing pupae and AnPe cells as in host cells such as Sf9 and High5, will readily make the advantageous characteristics of AnpeNPV vector system available to users of AcNPV vector system, which is the most extensively used BEV system in the world.

In this paper, we describe the nucleotide sequence of the AnpeNPV *p143* (putative DNA helicase) gene and the host range of the gene-introduced AcNPV.

Materials and Methods

Bacterium, insect cell lines and DNA

Competent *E. coli* strain XL1-Blue cells (Stratagene) were used for plasmid DNA transformations. Three lepidopteran insect cell lines, NISES-AnPe-428 (AnPe), IPLB-Sf9 (Sf9), and BTI-TN5B1-4 (High5) were maintained in TC-100 medium supplemented with 10% fetal bovine serum (FBS) (Sigma) at 27 ℃. *Pst*I P and H fragments of the AnpeNPV genome DNA cloned in pBluescriptII (Stratagene) (Huang *et al.*, 2002) were used for identification and nucleotide sequence determination of AnpeNPV *p143* gene. The 1.8 kbp *Eco*RI-*Sma*I cDNA fragment encoding a soluble form of enzymatically active human tissue non-specific alkaline phosphatase (hTNSALP), whose C-terminal portion from transmembrane domain to cytosolic tail was removed, was a gift from Prof. Dr. Kimimitsu Oda (School of Dentistry, Niigata University). A transfer vector plasmid pAcUW51 (Pharmingen) was used for construction of pAcAP and pAcAPhel as described later. BaculoGold linearized DNA (Pharmingen) was used as the AcNPV DNA genome.

DNA manipulations

All plasmid DNA recombination techniques were essentially as described by Sambrook *et al.* (1989). Restriction enzymes and other DNA modifying enzymes were purchased from Takara Bio.

DNA sequencing and sequence analysis

The nucleotide sequence of the AnpeNPV *p143* gene was determined using ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with the following oligonucleotide primers. The position and direction of each

primer except M13 forward and M13 reverse was shown in Fig. 1.

M13 forward: 5'-CGTTGTAAAACGACGGCCAG-3'
M13 reverse: 5'-CAGGAAACAGCTATGACCAT-3'
HEL1: 5'-ATCCAATTCTCCCGACCGAC-3'
HEL2: 5'-AACAAGTCTTCGCTCTTGTC-3'
HEL3: 5'-TTCCATTGTTGGTGCTAGGC-3'
HEL4: 5'-CAACGACAAGCGGTTCCGAG-3'
HEL5: 5'-TGCCTCTGTCCGGCAAATCG-3'
HEL6: 5'-TTGAGGATGTAGATGAGCGC-3'
HEL7: 5'-CGCGCAGCTTTGTGTTTGAC-3'
HEL8: 5'-CGTGAATTTGGCCACAGTGT-3'
HEL9: 5'-ACGCAGCTCACGCGCCTTTC-3'
APHX490: 5'-CTTTGGCCGAAAAGGACAAA-3'
APHE120: 5'-GTTTGATCTCGACAGCACAC-3'
APHE510: 5'-AACCGCAAACACGCCCACTT-3'
APHP430: 5'-ATTGACGCGCATCATGGTCT-3'

Homology search of predicted amino acid sequence of the AnpeNPV *p143* gene product was carried out using the basic local alignment search tool (BLAST) program provided by the GenomeNet WWW Server (Bioinformatics Center, Kyoto University). The GENETYX program (Genetyx) was used for both sequence data analysis and phylogenetic tree construction by the unweighted pair-group method with arithmetic mean (UPGMA).

Construction of recombinant AcNPVs

For the construction of recombinant AcNPVs, we modified the transfer vector pAcUW51 as follows (Fig. 2). The 1.8-kbp of *Eco*RI-*Sma*I fragment containing hTNSALP cDNA was inserted in pAcUW51 at *Eco*RI site just downstream of the AcNPV p10 promoter by blunt-end ligation and designated as pAcAP. The *p143* gene of AnpeNPV separated in *Pst*I H and P fragments of AnpeNPV DNA was combined by cloning both 1.9-kbp *Sal*I-*Pst*I portion of the *Pst*I H, containing the promoter and N-terminal coding region, and 3.1-kbp *Pst*I-*Nco*I portion of the *Pst*I P, containing the C-terminal coding region and polyA site, in pBluescriptII. Then, the entire AnpeNPV *p143* gene excised from the plasmid as 5.0-kbp *Sal*I-*Bam*HI fragment was cloned in the recombinant transfer vector pAcAP in place of polyhedrin promoter and designated as pAcAPhel as illustrated in Fig. 2.

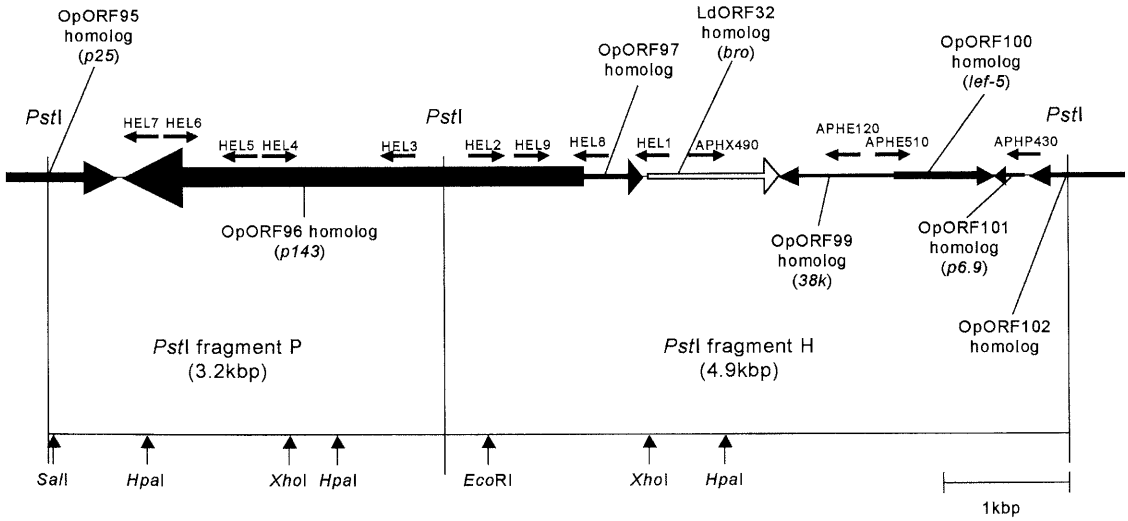


Fig. 1. Gene arrangement map of the AnpeNPV *Pst*I P and H fragments. The positions and directions of the OpMNPV ORF (OpORF) 95 (*p25*), 96 (*p143*), 97, 99 (*38k*), 100 (*lef-5*), 101 (*p6.9*) and 102 homologs are shown by black arrows and the those of LdNPV ORF (LdORF) 32 (*bro*) homolog are shown by a white arrow. Small arrows above the map indicate the positions and directions of the primers used for the nucleotide sequence determination and RT-PCR analysis as described in Materials and Methods. Recognition sites of several restriction enzymes are also indicated below the map. A scale bar represents 1 kbp.

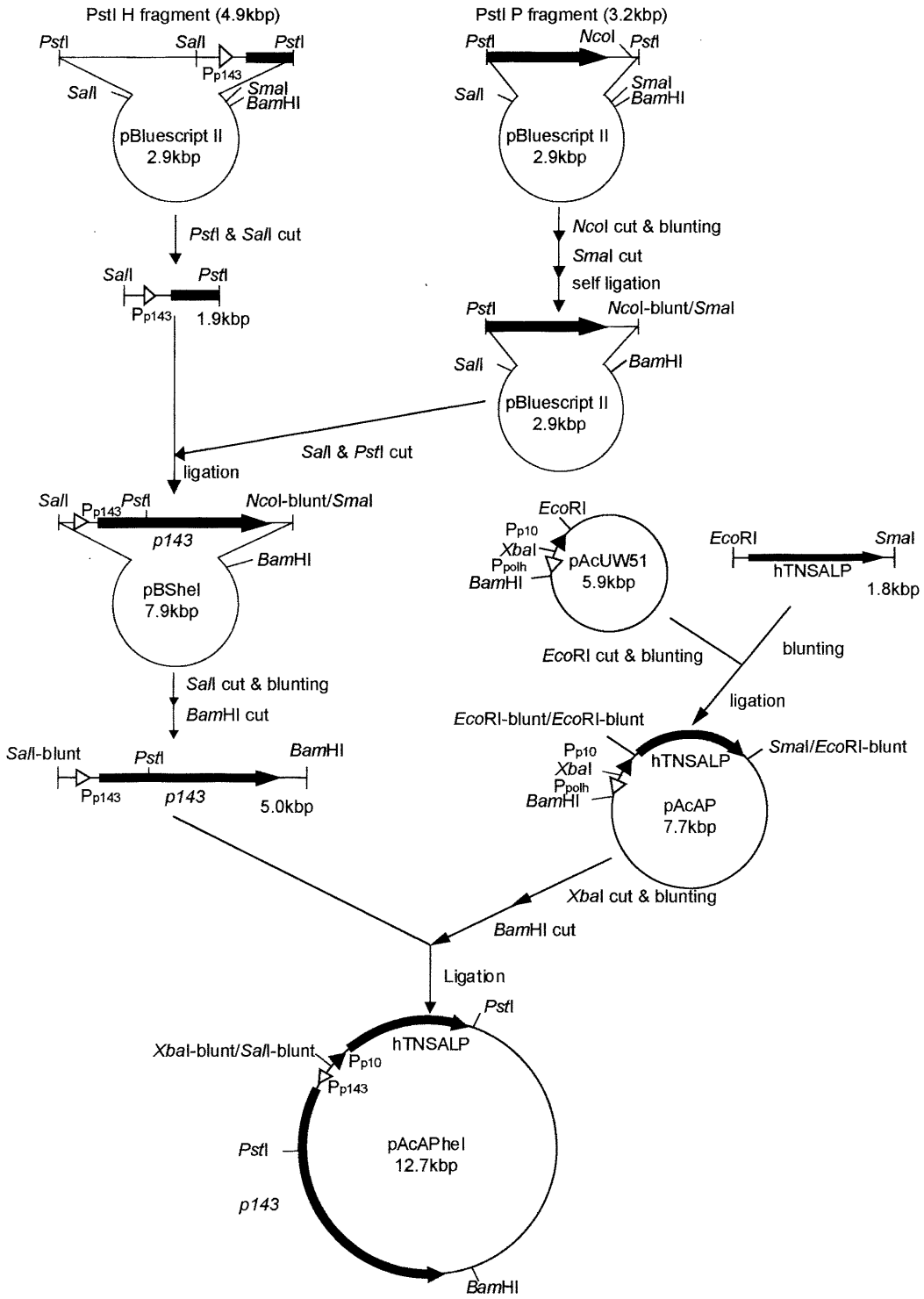


Fig. 2. Schematic diagram of procedure used for the construction of two recombinant transfer vector plasmids, pAcAP and pAcAPhel. The details are described in Materials and Methods.

To generate 2 recombinant AcNPVs, AcAP expressing the hTNSALP gene under the control of the AcNPV *p10* promoter and AcAPhel expressing both the AnpeNPV *p143* gene under the control of its own promoter and the hTNSALP gene under the control of the AcNPV *p10* promoter, pAcAP and pAcAPhel were respectively cotransfected with viral DNA (BaculoGold) into Sf9 cells by lipofection method, and recombinant viruses contained in culture supernatants of the transfected cells were purified by at least three rounds of plaque assay as described previously (Huang *et al.*, 2001). The insertion of the hTNSALP gene in AcAP and both the hTNSALP and AnpeNPV *p143* genes in AcAPhel at the *polyhedrin* lucus of each virus as well as the expression of hTNSALP gene were confirmed by PCR analysis of viral DNA and measuring alkaline phosphatase activity in the virus-infected culture supernatants, respectively.

Both AcAP and AcAPhel were amplified in Sf9 cells and, after measuring the virus titer, the infected culture supernatants were stored as virus stocks at -80℃ until use.

Virus infection and sample preparation

For measuring budded virus (BV) titer and alkaline phosphatase activity in the virus-infected culture supernatants, AnPe and Sf9 cells (1×10^6) in each well of 6-well plates (Sumitomo Bakelite) were infected with AcAP or AcAPhel at multiplicity of infection (MOI) = 5 for one hour and cultured in 2ml of TC-100 (+10% FBS) medium at 27℃ for 5 days. At 18 hours post-infection, 100 μl of the culture supernatants were harvested for determine the initial titer of BV in each well. At 5 days post-infection, all the culture supernatants were harvested and used for measuring both BV titer and alkaline phosphatase activity. For alkaline phosphatase assay, High5 cells were also used.

For analyzing the expression of both AnpeNPV and AcNPV *p143* genes in the virus-infected cells, AnPe, Sf9 and High5 cells (1×10^6) in T-25 flasks (Sumitomo Bakelite) were infected with AcAP or AcAPhel at multiplicity of infection (MOI) = 5 for one hour and cultured in 4 ml of TC-100 (+10% FBS) medium at 27℃ for 12 hours. Then the infected cells were harvested and mRNA were extracted using QuickPrep Micro mRNA Purification Kit (Amersham). After calculating RNA concentration by measuring absorbance at 260 nm, the mRNA samples were used as templates for RT-PCR amplification of cDNA fragments of both the AnpeNPV and AcNPV *p143* transcripts.

Virus titration

Sf9 cells (1×10^6) in each well of 6-well plates were inoculated with one of tenfold serial dilutions (1ml each) of the AcAP or AcAPhel-infected culture supernatntas for one hour. After removing inoculum, cells in each well were overlaid with 2.5 ml of TC-100 (+10% FBS) containing 0.75% SeaPlaque Agarose (BioWhittaker Molecular Applications) and cultured at 27℃ for 7 days. The number of plaques formed in each well was counted by microscopic observation to calculate the plaque forming unit (PFU) of BV contained in each culture supernatant.

Alkaline phosphatase assay

The alkaline phosphatase activity of hTNSALP in the infected culture supernatant was assayed using 10 mM *p*-nitrophenyl phosphate (PNPP) as a substrate in 0.2 M Glycine/NaOH buffer (pH 9.5) as follows. First, each culture supernatant properly diluted with 20 mM Tris-HCl (pH7.5) were mixed with PNPP in the buffer and absorbance at 405 nm (OD_{405}) was measured at both 15 min and 30 min after incubation at 37 ℃. Then, alkaline phosphatase activity (munit = nmol of PNPP hydrolyzed/min) per 1 ml of each culture supernatant was calculated by the following formula.

Alkaline phosphatase activity (munit/ml)=

$$\frac{(OD_{405}\text{ at 30 min} - OD_{405}\text{ at 15 min})\times V\text{ (ml)}\times d}{t\text{ (min)}\times v\text{ (ml)}\times \varepsilon\text{ (ml/nmol)}}$$

where V is the volume of a reaction mixture (3 ml), d is the dilution rate of culture supernatant, t is the reaction time (30 - 15 = 15 min), v is the volume of a sample (0.5 ml), and ε is the extinction

coefficient (16 ml/nmol).

RT-PCR analysis

The first strand cDNA was synthesized using cDNA Synthesis System (Invitrogen) with 0.1 μg of each mRNA sample extracted from virus-infected cells as a template. After 50 min of reverse transcription (RT) at 37 ℃, reaction was stopped by heating at 70℃ for 15 min. Then, 2 μl of each first strand cDNA synthesis mixture were subjected to PCR amplification of the *p143* cDNA fragments using ExTaq (Takara Bio) and the following primer pairs. The AnpeNPV *p143* cDNA fragment (969 bp) was amplified using a sense primer HEL3 and an antisense primer HEL4 (Fig. 1), which are corresponding to nt 1436 to 1455 and complimentary to nt 2385 to 2404 of the coding sequence, respectively, while the AcNPV p143 cDNA fragment (970 bp) was amplified using a sense primer ACHEL3 (5'-CCGCCTGACATTGTGTGTAA-3') and antisense primer ACHEL4 (5'-CCGATTCTTCAAACTGAACA-3'), which are corresponding to nt 1459 to 1478 and complimentary to nt 2409 to 2428 of the coding sequence, respectively. PCR was started by one cycle of 94℃ for 3 min, then followed by 30 cycles ofl min at 94℃, 2 min at 60℃ and 3 min at 72℃, and a terminal cycle of 7 min at 72℃ using PCR Thermal Cycler (Model TP240, Takara Bio). The RT-PCR products were analyzed by agarose gel electrophoresis.

Nucleotide sequence accession number

The nucleotide sequence of the total 8084-bp *Pst*I P and H fragments containing AnpeNPV *p25* (partial), *p143*, OpORF 97 homolog, *bro*, *38k*, *p6.9*, *lef-5*, and OpORF 102 homolog (partial) has been submitted to the DDBJ/EMBL/Genbank databases under accession number AB116659.

Results and Discussion

Nucleotide sequence determination of the AnpeNPV PstI P and H fragments

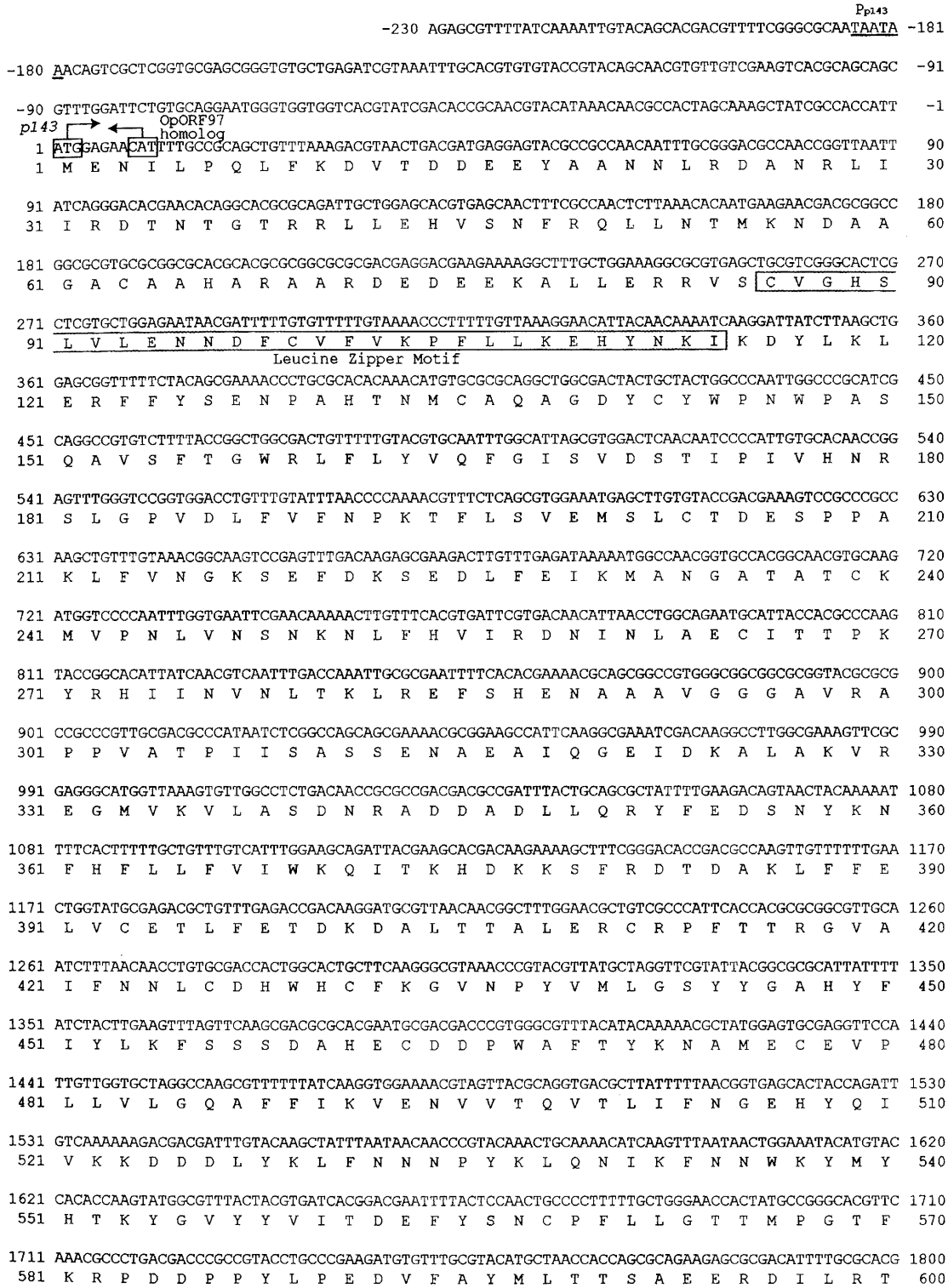
During the construction of a comparative genome map of AnpeNPV, we found partial sequences of the *p143* gene at the border between *Pst*I P and H fragments (Huang *et al.*, 2002). By constructing several deletion mutants and using the various primers described in Materials and Methods, complete nucleotide sequences of both 3.2-kbp *Pst*I P fragment and 4.9-kbp *Pst*I H fragment were determined. As shown in Fig. 1, 8 ORFs (6 complete and 2 partial ORFs) were found in the successive fragments. Homology search revealed that these 8 ORFs were homologs of OpMNPV ORFs 95 (*p25*), 96 (*p143*), 97, 99 (*39k*), 100 (*lef5*), 101 (*p6.9*) and 102 (Ahrens *et al.*, 1997) and *L. dispar* NPV (LdNPV) ORF 32 (bro) (Kuzio *et al.*, 1999), respectively. Relative positions and directions of the homologs of OpMNPV ORFs were well conserved in the AnpeNPV genome. However, no OpMNPV ORF 98 homolog but a homolog of LdNPV ORF 32 was found at the corresponding position of the AnpeNPV genome.

Sequence analysis of AnpeNPV p143 gene

The 3869-bp nucleotide sequence consists of 5' upstream and coding regions of the AnpeNPV *p143* gene was shown in Fig. 3. The gene encodes a polypeptide of 1212 amino acids.

Comparative analysis of the AnpeNPV *p143* upstream region with those of other group I NPVs revealed that the putative early promoter sequence TAATAA (Lu and Carstens, 1992; Ahrens and Rhormann, 1996) was also conserved at the corresponding position (nt -180 to -185) in the AnpeNPV genome (Fig. 3), although, like BmNPV, the late promoter element G/TTAAG found between the early promoter and the translation initiation codon in the AcNPV and OpMNPV *p143* upstream regions was not found in the corresponding region of AnpeNPV. As indicated in both the AcNPV and OpMNPV *p143* genes, immediately downstream of the AnpeNPV *p143* gene ATG is the initiation codon for a homolog of OpMNPV ORF 96 (Fig. 3).

In general, the product of baculovirus *p143* gene contains seven conserved motifs that show homology with domains conserved in members of a superfamily of DNA and RNA helicases (Lu & Carstens, 1991). The conserved motifs are located in the carboxyl terminal 320 amino acids (Lu & Carstens, 1991; Ahrens &


Pp143
-230 AGAGCGTTTTATCAAAATTGTACAGCACGACGTTTTCGGGCGCAATAATA -181
-180 AACAGTCGCTCGGTGCGAGCGGGTGTGCTGAGATCGTAAATTTGCACGTGTGTACCGTACAGCAACGTGTTGTCGAAGTCACGCAGCAGC -91
-90 GTTTGGATTCTGTGCAGGAATGGGTGGTGGTCACGTATCGACACCGCAACGTACATAAACAACGCCACTAGCAAAGCTATCGCCACCATT -1
p143
OpORF97
homolog
1 ATGGAGAACATTTTGCCGCAGCTGTTTAAAGACGTAACTGACGATGAGGAGTACGCCGCCAACAATTTGCGGGACGCCAACCGGTTAATT 90
1 M E N I L P Q L F K D V T D D E E Y A A N N L R D A N R L I 30
91 ATCAGGGACACGAACACAGGCACGCGCAGATTGCTGGAGCACGTGAGCAACTTTCGCCAACTCTTAAACACAATGAAGAACGACGCGGCC 180
31 I R D T N T G T R R L L E H V S N F R Q L L N T M K N D A A 60
181 GGCGCGTGCGCGGCGCACGCACGCGCGGCGCGCGACGAGGACGAAGAAAAGGCTTTGCTGGAAAGGCGCGTGAGCTGCGTCGGGCACTCG 270
61 G A C A A H A R A A R D E D E E K A L L E R R V S C V G H S 90
271 CTCGTGCTGGAGAATAACGATTTTTGTGTTTTTGTAAAACCCTTTTTGTTAAAGGAACATTACAACAAAATCAAGGATTATCTTAAGCTG 360
91 L V L E N N D F C V F V K P F L L K E H Y N K I K D Y L K L 120
Leucine Zipper Motif
361 GAGCGGTTTTTCTACAGCGAAAACCCTGCGCACACAAACATGTGCGCGCAGGCTGGCGACTACTGCTACTGGCCCAATTGGCCCGCATCG 450
121 E R F F Y S E N P A H T N M C A Q A G D Y C Y W P N W P A S 150
451 CAGGCCGTGTCTTTTACCGGCTGGCGACTGTTTTTGTACGTGCAATTTGGCATTAGCGTGGACTCAACAATCCCCATTGTGCACAACCGG 540
151 Q A V S F T G W R L F L Y V Q F G I S V D S T I P I V H N R 180
541 AGTTTGGGTCCGGTGGACCTGTTTGTATTTAACCCCAAAACGTTTCTCAGCGTGGAAATGAGCTTGTGTACCGACGAAAGTCCGCCCGCC 630
181 S L G P V D L F V F N P K T F L S V E M S L C T D E S P P A 210
631 AAGCTGTTTGTAAACGGCAAGTCCGAGTTTGACAAGAGCGAAGACTTGTTTGAGATAAAAATGGCCAACGGTGCCACGGCAACGTGCAAG 720
211 K L F V N G K S E F D K S E D L F E I K M A N G A T A T C K 240
721 ATGGTCCCCAATTTGGTGAATTCGAACAAAAACTTGTTTCACGTGATTCGTGACAACATTAACCTGGCAGAATGCATTACCACGCCCAAG 810
241 M V P N L V N S N K N L F H V I R D N I N L A E C I T T P K 270
811 TACCGGCACATTATCAACGTCAATTTGACCAAATTGCGCGAATTTTCACACGAAAACGCAGCGGCCGTGGGCGGCGGCGCGGTACGCGCG 900
271 Y R H I I N V N L T K L R E F S H E N A A A V G G G A V R A 300
901 CCGCCCGTTGCGACGCCCATAATCTCGGCCAGCAGCGAAAACGCGGAAGCCATTCAAGGCGAAATCGACAAGGCCTTGGCGAAAGTTCGC 990
301 P P V A T P I I S A S S E N A E A I Q G E I D K A L A K V R 330
991 GAGGGCATGGTTAAAGTGTTGGCCTCTGACAACCGCGCCGACGACGCCGATTTACTGCAGCGCTATTTTGAAGACAGTAACTACAAAAAT 1080
331 E G M V K V L A S D N R A D D A D L L Q R Y F E D S N Y K N 360
1081 TTTCACTTTTTGCTGTTTGTCATTTGGAAGCAGATTACGAAGCACGACAAGAAAAGCTTTCGGGACACCGACGCCAAGTTGTTTTTTGAA 1170
361 F H F L L F V I W K Q I T K H D K K S F R D T D A K L F F E 390
1171 CTGGTATGCGAGACGCTGTTTGAGACCGACAAGGATGCGTTAACAACGGCTTTGGAACGCTGTCGCCCATTCACCACGCGCGGCGTTGCA 1260
391 L V C E T L F E T D K D A L T T A L E R C R P F T T R G V A 420
1261 ATCTTTAACAACCTGTGCGACCACTGGCACTGCTTCAAGGGCGTAAACCCGTACGTTATGCTAGGTTCGTATTACGGCGCGCATTATTTT 1350
421 I F N N L C D H W H C F K G V N P Y V M L G S Y Y G A H Y F 450
1351 ATCTACTTGAAGTTTAGTTCAAGCGACGCGCACGAATGCGACGACCCGTGGGCGTTTACATACAAAAACGCTATGGAGTGCGAGGTTCCA 1440
451 I Y L K F S S S D A H E C D D P W A F T Y K N A M E C E V P 480
1441 TTGTTGGTGCTAGGCCAAGCGTTTTTTATCAAGGTGGAAAACGTAGTTACGCAGGTGACGCTTATTTTTAACGGTGAGCACTACCAGATT 1530
481 L L V L G Q A F F I K V E N V V T Q V T L I F N G E H Y Q I 510
1531 GTCAAAAAAGACGACGATTTGTACAAGCTATTTAATAACAACCCGTACAAACTGCAAAACATCAAGTTTAATAACTGGAAATACATGTAC 1620
521 V K K D D D L Y K L F N N N P Y K L Q N I K F N N W K Y M Y 540
1621 CACACCAAGTATGGCGTTTACTACGTGATCACGGACGAATTTTACTCCAACTGCCCCTTTTTGCTGGGAACCACTATGCCGGGCACGTTC 1710
551 H T K Y G V Y Y V I T D E F Y S N C P F L L G T T M P G T F 570
1711 AAACGCCCTGACGACCCGCCGTACCTGCCCGAAGATGTGTTTGCGTACATGCTAACCACCAGCGCAGAAGAGCGCGACATTTTGCGCACG 1800
581 K R P D D P P Y L P E D V F A Y M L T T S A E E R D I L R T 600

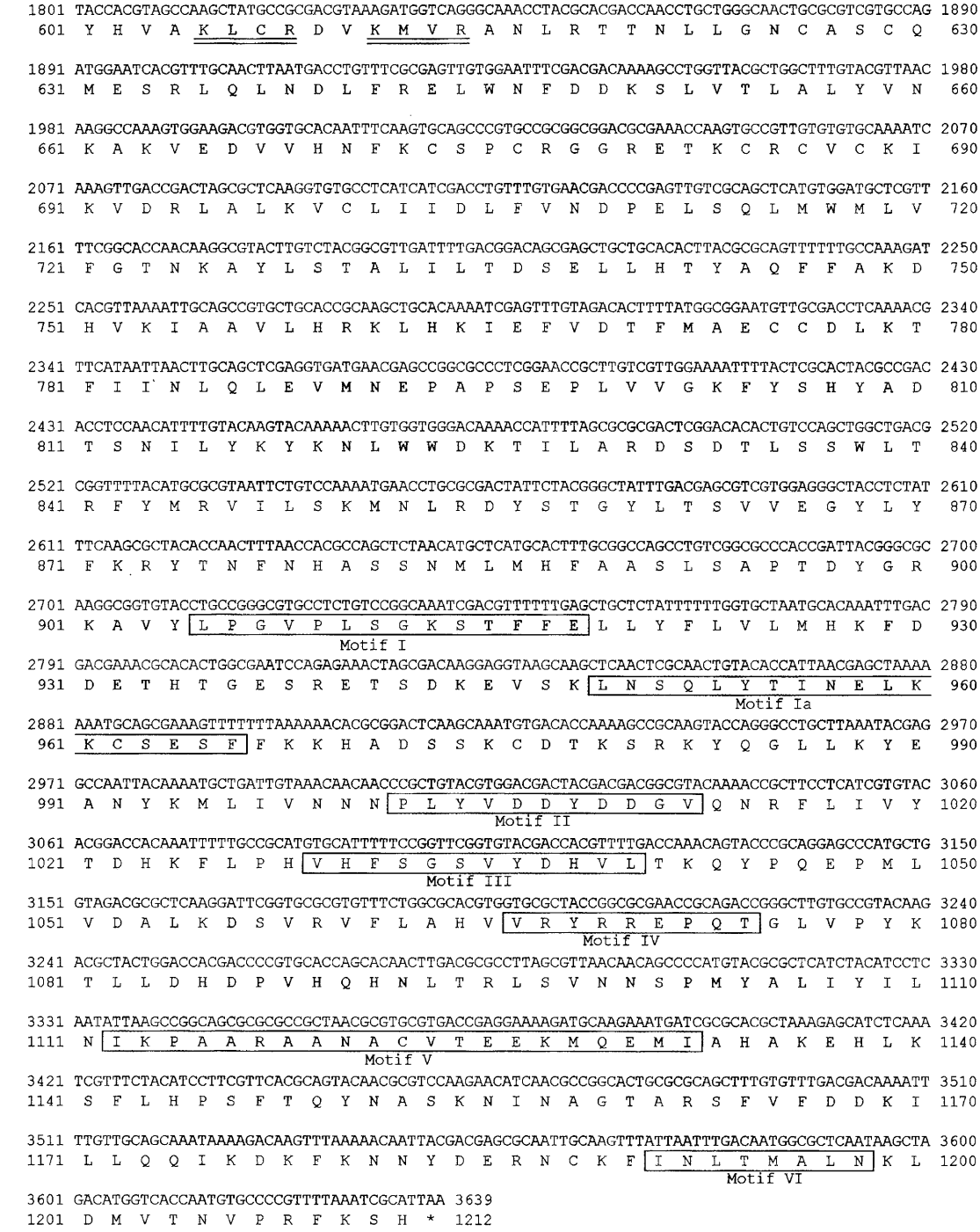


Fig. 3. Nucleotide sequence of the AnpeNPV *p143* gene region shown with the deduced amino acid sequence. In the nucleotide sequence, a putative early promoter sequence TAATAA of *p143* is underlined and translation initiation ATG codons of both *p143* and OpORF 97 homolog are boxed with an arrow indicating the direction of translation. In the amino acid sequence, 1 leucine zipper motif and 7 conserved helicase motifs (I, Ia, II, III, IV, V and VI) are boxed and 2 nuclear localization signals are double-underlined.

Rohrmann, 1996) and their linear spatial arrangement is identical to that found in other members of the helicase superfamily (N terminus-I-Ia-II-III-IV-V-VI-C terminus) (Gorbalenya *et al.*, 1989; Hodgman, 1988). In the case of the AnpeNPV *p143* gene, all of these conserved motifs have been identified in the carboxyl terminal portion (Fig. 3). In addition to these motifs, as identified in the putative DNA helicase (*p143* gene product) of AcNPV (Lu & Carstens, 1991), a leucine zipper motif (White and Weber, 1989) and two putative nuclear localization signals (NLS) with the core sequence KXXK/R (Roberts, 1989) were also conserved (Fig. 3). All of these characteristics in primary structure indicated that the AnpeNPV *p143* gene encodes a putative DNA helicase.

As shown in Fig. 4, amino acid sequence identities to putative DNA helicases of other baculoviruses varied between 78% (OpMNPV) and 25% (3 GVs), and a phylogenetic tree constructed on the basis of multiple alignments of putative DNA helicase sequences supported the separation of the group I NPVs, group II NPVs and GVs, and well consisted with another tree of baculovirus DNA helicase previously constructed by Herniou *et al.* (2001).

Effects of AnpeNPV ***p143*** *gene on host range of AcNPV*

To evaluate the ability of AnpeNPV *p143* gene to expand the host range of AcNPV, we constructed two recombinant AcNPVs (AcAP and AcAPhel) as described in Materials and Methods (Fig. 2), and compared their replication and protein production in Sf9, High5, and AnPe cells.

From mRNA samples of 3 cell lines at 12 hours post-infection (p.i.) of both AcAP and AcAPhel, 970-bp DNA fragments were amplified by RT-PCR using the AcNPV *p143* gene-specific primers ACHEL3 and ACHEL4 (Fig. 5A). In addition, from mRNA samples of AcAPhel-infected 3 cell lines at 12 hours p.i., 969-bp DNA fragments were amplified by RT-PCR with the AnpeNPV *p143* gene-specific primers HEL3 and HEL4 (Fig. 5B). These DNA fragments were not amplified by PCR without the preceding RT reaction, indicating that not only host cell lines of AcNPV (Sf9 and High5) but also a non-host cell line (AnPe) permit entry of AcNPV and expression of both the AcNPV and AnpeNPV *p143* genes from their respective early promoters, although their expression levels were not compared quantitatively by this RT-PCR analysis.

BV titers in culture supernatants of AnPe cells infected with AcAP and AcAPhel increased 250-fold and 50-fold from 18 hours p.i. to 5 days p.i. respectively (Table 1). These BV proliferation rates were less than one tenth of those for Sf9 cells. Therefore, AnPe cells are not completely nonpermissive for the AcNPV infection but semipermissive. The results also revealed that the AnpeNPV *p143* gene introduced in AcAPhel genome did not improve the BV production in AnPe cells.

Alkaline phosphatase activities in culture supernatants of AnPe cells infected with AcAP and AcAPhel were also less than one tenth of those for Sf9 cells (Fig. 6). The AnpeNPV *p143* gene introduced in AcAPhel genome did not improve the alkaline phosphatase production in AnPe cells but those in Sf9 and High5 cells drastically. The results might suggest that the AnpeNPV *p143* gene functions as a enhancer of the AcNPV *p10* very late promoter in the host cells such as Sf9 and High5, although there is another possibility that removal of another very late *polyhedrin* promoter from just upstream of the *p10* promoter in the transfer vector pAcAPhel (Fig. 2) might indirectly enhance the *p10* promoter activity by eliminating competition between the two very late promoters.

As described above, AcNPV expressed its own *p143* gene at early phase of virus infection in both permissive Sf9 and High5 cells and a semipermissive AnPe cells, while BV production at late phase and recombinant protein (alkaline phosphatase) production at very late phase were suppressed to lower levels in AnPe cells. The AnpeNPV *p143* gene introduced in AcNPV did not enhance either BV production or recombinant protein production in AnPe cells, although the introduced gene expressed at early phase of infection. Thus, the AnpeNPV *p143* gene was not effective in host range expansion of AcNPV to semipermissive AnPe

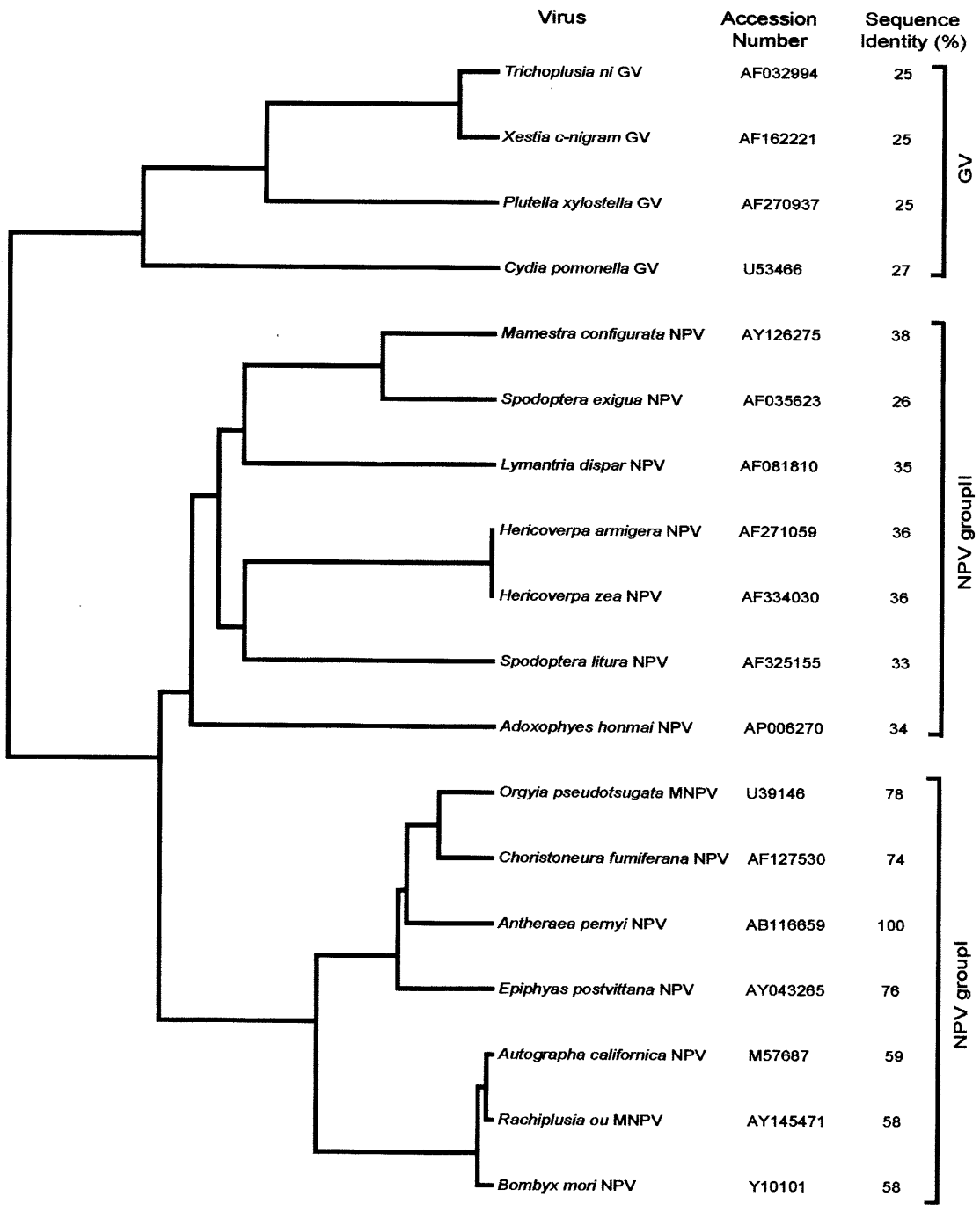


Fig. 4. A phylogenetic tree of baculoviruses constructed on the basis of the multiple alignment of amino acid sequences of putative DNA helicase using the unweighted pair-group method with the arithmetic mean (UPGMA) procedure with the GENETYX program. The virus names are indicated with the accession number of sequence data and sequence identities (%) to the AnpeNPV *p143* gene product. The classification of baculoviruses into NPV group I, group II and GV is also indicated.

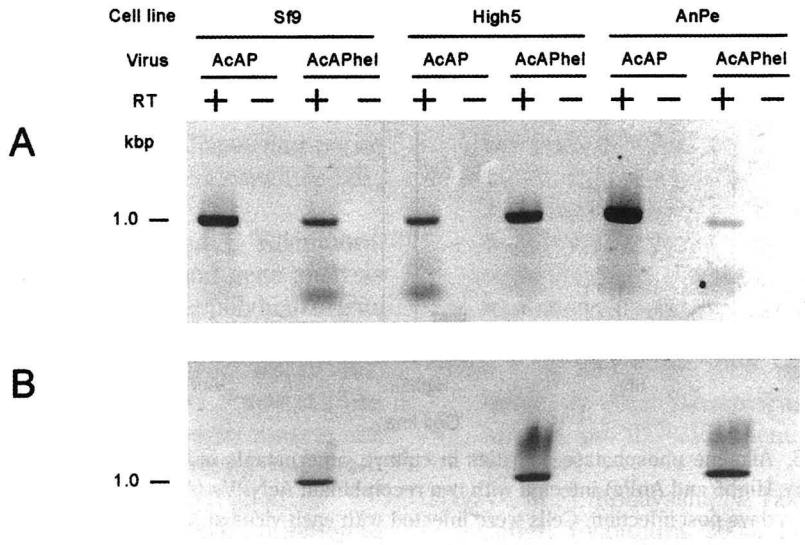


Fig. 5. RT-PCR analyses of the *p143* gene expression in 3 insect cell lines (Sf9, High5 and AnPe) infected with two recombinant AcNPVs (AcAP and AcAPhel) at 12 hours post-infection. (A): AcNPV *p143* cDNA fragments (970 bp) amplified with primers ACHEL3 and ACHEL4. (B): AnpeNPV *p143* cDNA fragments (969 bp) amplified with primers HEL3 and HEL4. RT + and - indicate RT-PCR and PCR without RT, respectively. Cells were infected with each virus at MOI = 5.

Table 1. Comparison of virus titers in culture supernatnats of recombinant AcNPV-infected cells

Virus	Cell line	Virus titer ($\times 10^3$ PFU/ml)		Proliferation rate (B/A)
		18 hours p.i. (A)	5 days p.i. (B)	
AcAP	SF9	14	52000	3700
	AnPe	4	1000	250
AcAPhel	Sf9	28	46000	1600
	AnPe	8	400	50

Cells were infected with each virus at MOI=5.

cells. Similarly, incorporation of *T. ni* GV (TnGV) DNA helicase gene (*p137*) to *p143*-deficient AcNPV failed to support the replication of AcNPV in both High5 cells and *T. ni* larvae, and co-expression of the TnGV *p137* and AcNPV *p143* genes did not inhibit the AcNPV replication (Bideshi and Federici 2000). However, our result is not conclusive, because functions of the AnpeNPV *p143* gene might be inhibited by the AcNPV *p143* gene co-expressed by AcAPhel, as occurred in BmN cells co-infected with AcNPV and BmNPV (Kamita and Maeda, 1993). Or introduction of other host range-related genes such as the AnpeNPV iaps may enable the productive replication of AcNPV in AnPe cells. Further studies will revealed that either possibilities is true.

Acknowledgments

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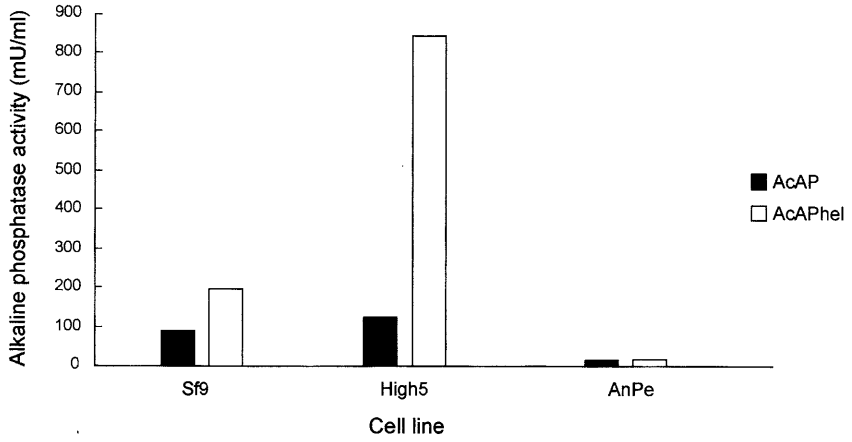

Fig. 6. Alkaline phosphatase activities in culture supernatants of 3 insect cell lines (Sf9, High5 and AnPe) infected with two recombinant AcNPVs (AcAP and AcAPhel) at 5 days post-infection. Cells were infected with each virus at MOI = 5.

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