Int. J. Indust. Entomol. Vol. 3, No. 1, 2001, pp. 25~29

International Journal of *Industrial Entomology*

Molecular Cloning of the Antiapoptotic Gene, *p35*, from *Bombyx mori* Nuclear Polyhedrosis Virus K1

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(Received 28 June 2001; Accepted 17 July 2001)

We have cloned and characterized an antiapoptotic gene, *p35*, which blocks apoptosis, from *Bombyx mori* nuclear polyhedrosis virus (BmNPV) K1 strain. The 897 bp *p35* has an open reading frame of 299 amino acids. The BmNPV-K1 *p35* showed a high identity to *Autographa californica* nuclear polyhedrosis virus and BmNPV T3 strain. The BmNPV-K1 *p35* was different from the amino acid sequences of BmNPV T3 at 6 positions. The *p35* gene of BmNPV-K1 was 99.2% identical at the nucleotide level and 98% identical at the amino acid level to BmNPV T3. The location of *p35* gene in the BmNPV-K1 genome was confirmed by Southern blot analysis and its expression patterns at the transcriptional level in the infected cells were confirmed by Northern hybridization analysis.

Key words : Baculovirus, *Bombyx mori* nuclear polyhedrosis virus, Antiapoptotic gene (*p35*)

Introduction

Baculoviruses possess a large circular DNA genome that replicates in the nuclei of infected cells. During infection, baculovirus genes are expressed in a highly regulated cascade in which early gene expression and viral replication are essential for late and very late gene expression. Apoptosis appears to be important as a cellular defense against virus infection, and large DNA-containing viruses carry genes involved in blocking cellular apoptosis either at the signal transduction level or at the commitment stage (Clem and Miller, 1994). Baculoviruses have two different types of genes which are capable of preventing cellular apoptosis during virus infection: antiapoptotic gene, *p35* and inhibitor of apoptosis gene, *iap* (Birnbaum *et al.*, 1994; Clem *et al.*, 1991; Crook *et al.*, 1993; Hershberger *et al.*, 1992; Kamita *et al.*, 1993).

Autographa californica nuclear polyhedrosis virus (AcNPV) contains an antiapoptotic gene, *p35* (Clem *et al.*, 1991; Hershberger *et al.*, 1992). The product of the early gene *p35* is required for AcNPV replication in *Spdoptera frugiperda* cell line SF-21. AcNPV lacking *p35* induces extensive apoptosis in SF-21 cells (Clem *et al.*, 1991; Hershberger *et al.*, 1992), and an arrest of protein synthesis was reported in the apoptotic SF-21 cells (Birnbaum *et al.*, 1994; Clem and Miller, 1993). AcNPV-induced apoptosis, including the activation of caspases, membrane blebbing, and DNA fragmentation, coincides with the initiation of the late phase of infection (Clem *et al.*, 1991; LaCount and Friesen, 1997). The product of *p35* gene functions by inhibiting the activity of caspases and thus prevents caspase-induced apoptosis (Bertin *et al.*, 1996; Bump *et al.*, 1995). In AcNPV, apoptosis significantly reduces budded virus production and completely eliminates occluded virus formation in SF-21 cells and thus is considered to be an effective host defense response against viral infection (Clem and Miller, 1993; Hershberger *et al.*, 1992). The *p35* is found in AcNPV and *Bombyx mori* NPV (BmNPV) (Friesen and Miller, 1987; Kamita *et al.*, 1993), and the P35 protein is known to be able to inhibit cell death in a great number of organisms and situations (Sugimoto *et al.*, 1994; Hay *et al.*, 1994; Beidler *et al.*, 1995; Rabizadeh *et al.*, 1993).

AcNPV and BmNPV are extensively studied members of baculovirus. These NPVs have been utilized in the studies of virus genetic structure, gene expression, development of baculoviruses as expression vectors of foreign genes, and genetically modified virus insecticides (Ayres

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et al., 1994; Gomi *et al.*, 1999; King and Possee, 1992; OReilly *et al.*, 1992). In BmNPV, T3 strain has been studied extensively (Gomi *et al.*, 1999; Maeda, 1984; Maeda *et al.*, 1985), but Korean strain K1, which is slightly different from the BmNPV T3 in viral genome, is not well understood. The polyhedrin (Woo *et al.*, 1995), *p10* (Kang *et al.*, 1997), *ie1* (Park *et al.*, 2001a), *vlf-1* (Park *et al.*, 2000), *egt* (Park *et al.*, 2001b) genes from BmNPV-K1 were identified and developed into polyhedrin gene- and *p10* gene-based expression vectors (Kang *et al.*, 1997; Woo *et al.*, 1995).

In this study, we have cloned and characterized the anti-apoptotic gene *p35* from BmNPV-K1. The sequence of BmNPV-K1 *p35* presented here was aligned to that of AcNPV (Friesen and Miller, 1987) and BmNPV T3 (Kamita *et al.*, 1993).

Materials and Methods

Cells and virus

The *Bombyx mori* 5 (Bm5) (Grace, 1962) cells were grown at 27°C in TC-100 medium (GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL) (O'Reilly *et al.*, 1992). Wild-type BmNPV-K1 (Kang *et al.*, 1997; Park *et al.*, 2000; Woo *et al.*, 1995) was propagated and titered in Bm5 cells. The titer was expressed as plaque forming units (PFU) per ml (O'Reilly *et al.*, 1992).

Viral genome isolation and PCR

Polyhedra and viral DNA were obtained from Bm5 cells by standard methods (O'Reilly *et al.*, 1992). Viral DNAs were used as templates. The p35 gene was amplified from viral DNAs using the primers 5-GAGCATTTGAGCTTTACCATTGC-3 and 5-TGTTAGTTCGTTACTGTT-3, annealing to the translation start region and translation termination region, respectively (Friesen and Miller, 1987; Kamita *et al.*, 1993). After 35-cycle amplification (94°C for 1 min; 55°C for 1 min; 72°C for 1 min), PCR product was analyzed by 1% agarose gel electrophoresis.

DNA sequencing

The PCR product was purified with PCR purification kit (QIAGEN) following manufacturers instruction and then cloned into pGem-T vector (Promega). The deletion mutants of *p35* gene were constructed using an Exo Mung Bean Deletion Kit (Stratagene). DNA sequencing was performed using an automatic sequencer (model 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, CA). Sequence alignment was performed using IBI MacVector (ver. 6.5).

Southern blot analysis

Viral DNAs digested with *Cla*I and *Eco*RI were electrophoresed through a 1.0% agarose gel as described previously (OReilly *et al.*, 1992). The DNA from the gel was transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42°C with a probe in a hybridization buffer containing 5 × SSC, 50% formamide, 0.1% (W/V) *N*-lauroylsarcosine, 0.02% sodium dodecyl sulphate (SDS) and 2% blocking agent (Boehringer Mannheim, Mannheim, Germany). The probe used to detect the DNA fragment containing *p35* gene was a 0.9 kb BmNPV-K1 p35 gene radiolabeled with [α-^{32}P] dCTP (Amersham, Arlington Heights, IL). After hybridization, the membrane filter was washed three times for 30 min each in 0.1% SDS and 0.2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C, and finally exposed to *X*-ray film.

RNA isolation and Northern blot analysis

Total cellular RNA was isolated from mock-infected or wild-type BmNPV-infected Bm5 cells. A total of 1×10^6

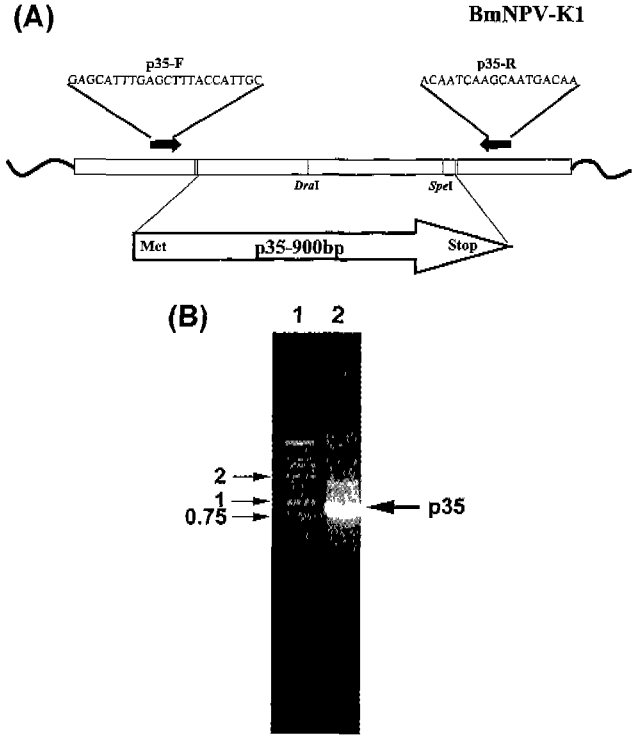

Fig. 1. PCR of *p35* gene from BmNPV-K1. The PCR primers for identification of BmNPV-K1 p35 were based on the previously identified *p35* of AcNPV (Friesen and Miller, 1987) and BmNPV T3 (Kamita *et al.*, 1993) (A). The amplified PCR product was analyzed by 1% agarose gel electrophoresis (B). Lane 1, molecular size marker; lane 2, BmNPV-K1. Arrow indicates the amplified *p35* from BmNPV-K1.

cells per 35-mm-diameter dish was infected at a multiplicity of infection of 5 PFU per cell. Cells were collected at 4, 8, 12, 18, 24, 36, and 48 hrs postinfection (p.i.). Total cellular RNA was isolated using Total RNA extraction kit (Promega). Total cellular RNA (10 μg per lane) from the infected cells was denatured by glyoxalation (McMaster and Carmichael, 1977), transferred onto a nylon blotting membrane (Schleicher & Schuell) and hybridized at 42°C with a probe in a buffer containing 2 × PIPES, 50% formamide, 1% SDS and blocking agent (Boehringer Mannheim). The probe used to detect the p35 gene transcripts was a 0.9 kb BmNPV-K1 *p35* gene radiolabeled with [α-^{32}P] dCTP (Amersham). The other procedures for washing the membrane filter and exposing *X*-ray film were performed by the Southern blot analysis described above.

Results and Discussion

When the nucleotide sequences of the BmNPV T3 (Gomi *et al.*, 1999) and AcNPV genomes (Ayres *et al.*, 1994) were compared, ORFs were highly conserved (over 90% identity). The average amino acid sequence identity between homologous ORFs was about 93% (Gomi *et al.*, 1999). To identify *p35* gene in BmNPV-K1, therefore, we have employed PCR by designing primer set based on the conserved region of *p35* of AcNPV and BmNPV T3 (Fig. 1A). The expected amplified PCR product was amplified in BmNPV-K1 (Fig. 1B). As shown in Fig. 1, the molecular size of the product in BmNPV-K1 was identical to that expected. The PCR product for sequencing was cloned.

The nucleotide sequence of PCR product was analyzed

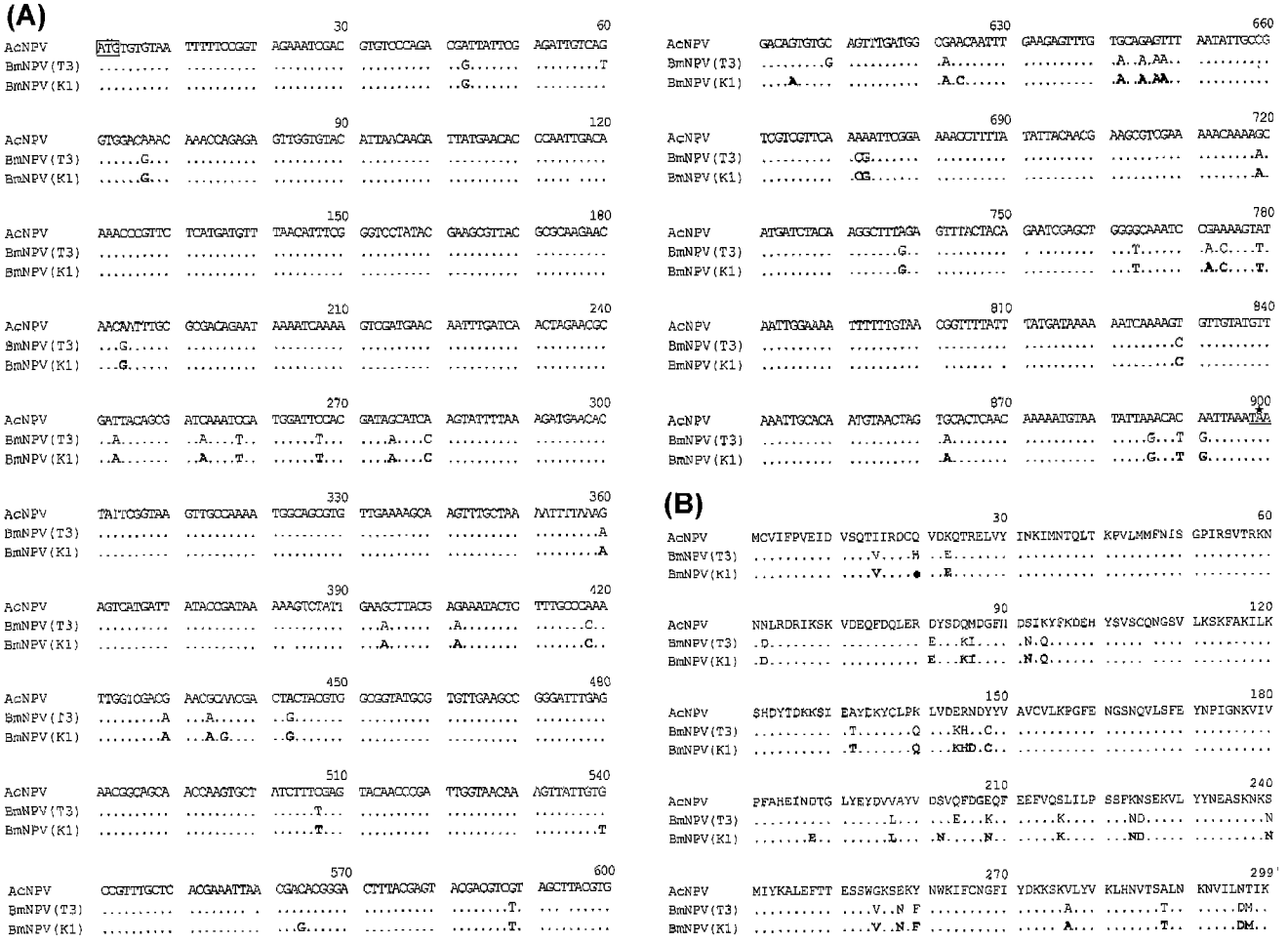

Fig. 2. Nucleotide (A) and deduced amino acid (B) sequences of BmNPV-K1 *p35*. The sequences of BmNPV-K1 were compared with those of AcNPV and BmNPV T3. The translation initiation codon (open box) and translation termination codon (asterisk) of *p35* are indicated. The differences among BmNPV-K1, AcNPV and BmNPV T3 sequences are indicated in boldface at nucleotide and amino acid sequence positions. Identical sequences are indicated by dots below the AcNPV sequence. The sequence of BmNPV-K1 has been deposited in GenBank (Accession number AY048772).

Table 1. Alignment of the nucleotide sequence of the *p35* coding region from BmNPV-K1

	1	2	3
AcNPV	-	4.22	4.55
BmNPV T3	38	-	0.78
BmNPV-K1	41	7	-

Numbers above the diagonal are mean distance values; numbers below the diagonal are absolute distance values.

Table 2. Alignment of the amino acid sequence of the *p35* coding region from BmNPV-K1

	1	2	3
AcNPV	-	9.36	9.69
BmNPV T3	28	-	2.00
BmNPV-K1	29	6	-

Numbers above the diagonal are mean distance values; numbers below the diagonal are absolute distance values.

and its amino acid was deduced. As the result of the complete nucleotide sequence (GenBank accession number; AY048772) in Fig. 2, the *p35* of 897 bp has an open reading frame of 299 amino acids with MW of about 35 kDa. The nucleotide and deduced amino acid sequences of BmNPV-K1 *p35* were compared with those of AcNPV and BmNPV T3, respectively. When the BmNPV-K1 *p35* and BmNPV T3 *p35* are aligned, nucleotide and amino acids sequence homologies amounted to 99.2% and 98%, respectively (Table 1 and 2). The nucleotide sequences of BmNPV-K1 *p35* differ 7 positions from BmNPV T3. In addition, BmNPV-K1 p35 differ 6 amino acid positions (20, 147, 188, 202, 204, and 208) from BmNPV T3. The sequences of the nucleotide and amino acid of the BmNPV-K1 are 95.5% and 90.3% identical to those of AcNPV, demonstrating a high identity among them (Friesen and Miller, 1987; Kamita *et al.*, 1993).

The location of *p35* gene in the BmNPV-K1 genome was confirmed by Southern blot analysis. BmNPV-K1 genome was digested with ClaI and EcoRI, and probed with the PCR-amplified *p35* (Fig. 3). The *p35* in BmNPV-K1 genome was located on the 4.0 kb *Cla*I fragment and 2.2 kb *Eco*RI fragment.

To verify whether the *p35* transcripts were correlated with virus replication, we examined the virus-infected cells by Northern blot analysis with *p35* probe (Fig. 4). Total cellular RNA purified from Bm5 cells 4, 8, 12, 18, 24, 36 and 48 hrs p.i. with wild-type BmNPV-K1 was hybridized with an excess of probe. As shown in Fig. 4, *p35* transcripts were detected at 4 hrs p.i., and maximally observed at 12 and 18 hrs p.i. The *p35* transcripts were maintained during 48 hrs p.i., but slightly decreased at 24 hrs p.i. This result is consistent with the previous result

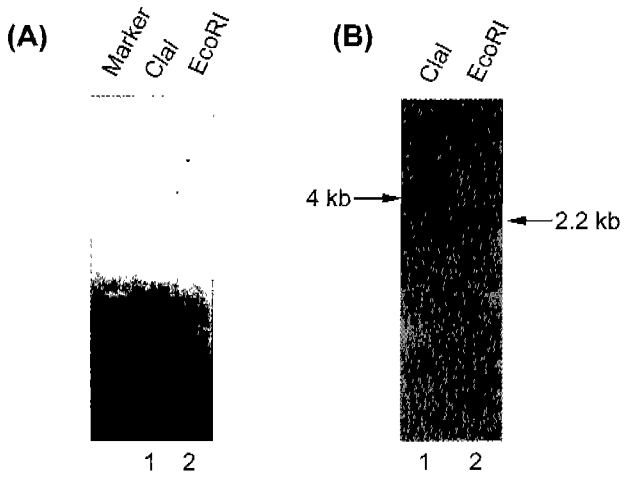

Fig. 3. Southern blot analysis of BmNPV-K1 genome. Viral DNAs digested with *Cla*I (lane 1) and *Eco*RI (lane 2) were electrophoresed through a 1.0% agarose gel (A) and hybridized at 42°C with a labeled probe (B). The probe used to detect DNA fragment containing *p35* was a 0.9 kb BmNPV-K1 *p35* amplified by PCR in this study. Hybridized bands are indicated by arrow with molecular size.

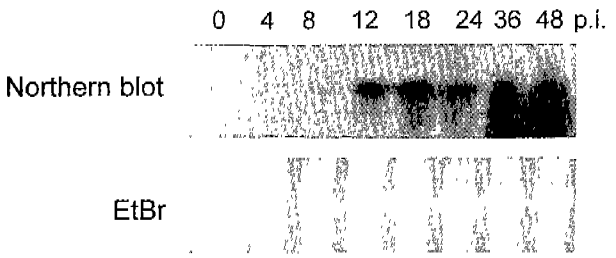

Fig. 4. Northern blot analysis of *p35* transcripts from BmNPV-K1-infected cells. Total RNA was collected from Bm5 cells at various times p.i. as indicated at the top of each lane. The probe used to detect *p35* transcripts was a 0.9 kb BmNPV-K1 *p35* amplified by PCR in this study.

that P35 protein in SF-21 cells infected with AcNPV was detected from 6 upto 48 hrs p.i. (Du and Thiem, 1997).

In conclusion, we have cloned and characterized a novel *p35* gene from the BmNPV-K1. Knowledge of the *p35* in this study will provide the genetic information for establishing BmNPV-K1 strain.

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