Int. J. Indust. Entomol. Vol. 3, No. 1, 2001, pp. 43~49

International Journal of *Industrial Entomology*

Molecular Cloning and Sequence Analysis of the Immediate Early Viral Gene, *IE1*, from *Bombyx mori* Nuclear Polyhedrosis Virus K1

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(Received 21 June 2001; Accepted 29 July 2001)

We have cloned and characterized an immediate early-1 gene, *ie1*, which is activated immediately upon entrance of the viral genome into the cell nucleus, from *Bombyx mori* nuclear polyhedrosis virus (BmNPV) K1 strain. This gene encodes a protein 584 amino acids with a predicted molecular weight of 67 kDa. The promoter and coding regions of BmNPV-K1 *ie1* showed high homology with *Autographa californica* nuclear polyhedrosis virus and BmNPV T3 strain. The BmNPV-K1 *ie1* was different from amino acid sequence at 4 positions in BmNPV T3. The location of *ie1* 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, Immediate early 1 gene (*ie1*)

Introduction

Baculoviruses have large, circular, double-stranded DNA genomes and are particularly pathogenic for insects of the order Lepidoptera. Nuclear polyhedrosis virus (NPV), member of a genus of the family Baculoviridae, is the main virus most frequently used for studies of baculovirus. Among these NPVs, *Autographa californica* NPV (AcNPV) and *Bombyx mori* NPV (BmNPV) are widely studied members of baculovirus. These NPVs have been utilized in studies of the virus genetic structure, gene expression, development of baculoviruses as expression vectors of foreign genes, and genetically modified virus insecticides (Ayres *et al.*, 1994; Gomi *et al.*, 1999; King and Possee, 1992; Maeda *et al.*, 1985; OReilly *et al.*, 1992; Smith *et al.*, 1983).

Viral gene expression of NPVs in infected cells is tightly regulated and occurs in a cascade fashion (Blissard and Rohrmann, 1990; Friesen and Miller, 1986). The viral gene expression is subdivided into early and late phases. Early-phase viral genes are transcribed by the host RNA polymerase II (Huh and Weaver, 1990) and encode proteins that are required for DNA replication and late-gene transcription. At least four early genes encode proteins that activate transcription of other early promoters (Carson *et al.*, 1988; Guarino and Summers, 1986; Kovacs *et al.*, 1991; Lu and Carstens, 1994). The major transcriptional regulator of NPVs is an immediate-early protein 1 (IE1). IE1 was initially characterized as a potent transcriptional activator of early genes (Guarino and Summers, 1986; Ribiero *et al.*, 1994) and is essential for both DNA replication and late transcription (Kool *et al.*, 1994; Passarelli and Miller, 1993).

IE1 homologues have been identified in AcNPV (Guarino and Summers, 1987; Ribeiro *et al.*, 1994), *Orgyia pseudotsugata* NPV (Theilmann and Stewart, 1991), BmNPV (Huybrechts *et al.*, 1992), and *Helicoverpa zea* NPV (Cowan *et al.*, 1994). Jarvis *et al.* (1990) demonstrated that the IE1 promoter could be used to produce transformed lepidopteran cells that will express a foreign gene product continuously in the absence of viral infection. The generation of transformed lepidopteran cells expressing foreign proteins continuously has also been reported (Parrell *et al.*, 1998; Jarvis *et al.*, 1990; Joyce *et al.*, 1993; Park *et al.*, 2001a).

In BmNPVs, one of the well-known strains is a BmNPV T3, which was originally isolated by Maeda (1984). The BmNPV T3 strain has been studied extensively (Gomi *et al.*, 1999; Maeda *et al.*, 1985), but Korean strain K1,

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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), *egt* (Park *et al.*, 2001b), *vlf-1* (Park *et al.*, 2000), *p35* (Lee *et al.*, 2001) genes from BmNPV-K1 were identified and developed 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 ie1 gene from BmNPV-K1. The sequence of BmNPV-K1 *ie1* gene presented here was aligned to that of AcNPV (Guarino and Summers, 1987; Ribeiro *et al.*, 1994) and BmNPV T3 (Huybrechts *et al.*, 1992).

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 LIFE Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (GIBCO BRL LIFE Technologies) (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

Polyhedra and viral DNA were obtained from Bm5 cells by standard methods (O'Reilly *et al.*, 1992). Polyhedra were purified by centrifugation through discontinuous 40 to 65% sucrose gradients. Viral DNA was isolated from purified polyhedra by proteinase K digestion followed by phenol extraction (O'Reilly *et al.*, 1992).

Polymerase chain reaction (PCR)

Viral DNAs were used as templates. The *ie1* gene was amplified from viral DNAs using the primers 5-ATCGATGTCTTTGTGATGCG-3 and 5-GAACATGATTACACCTCCG-3, annealing to the 5 promoter region and 3 untranslational region respectively (Guarino and Summers, 1987; Huybrechts *et al.*, 1992; Ribeiro *et al.*, 1994). 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, Madison, WI). The deletion mutants of *ie1* 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, Foster City, 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 (O'Reilly *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 *ie1* gene was a 3.1 kb BmNPV-K1 *ie1* 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

Total cellular RNA was isolated from mock-infected or

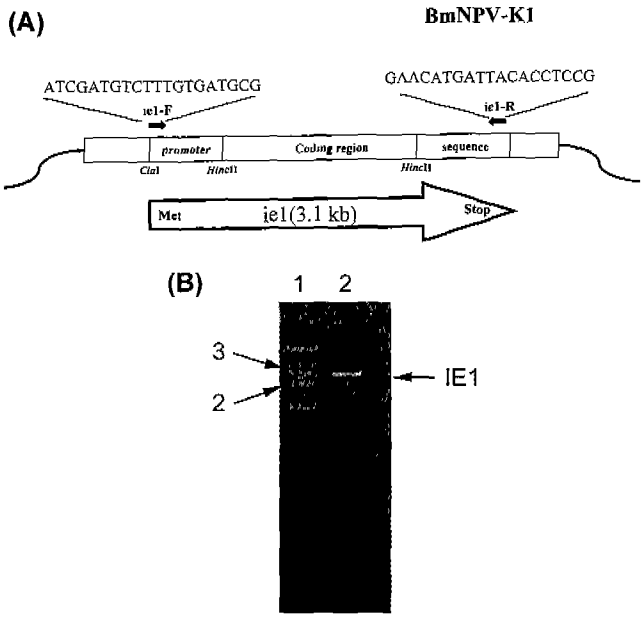

Fig. 1. PCR of *ie1* gene from BmNPV-K1. The PCR primers for identification of BmNPV-K1 *ie1* were based on the previously identified *ie1* genes of AcNPV (Guarino and Summers, 1987) and BmNPV T3 (Huybrechts *et al.*, 1992) (A). The amplified PCR product was analyzed by 1% agarose gel electrophoresis (B). Lane 1, molecular size marker; lane 2, BmNPV-K1. Arrow indicates the amplified ie1 gene from BmNPV-K1.

Fig. 2. Nucleotide (A) and deduced amino acid (B) sequences of BmNPV-K1 *ie1* gene. The sequences of BmNPV-K1 *ie1* were compared with those of AcNPV and BmNPV T3. The open reading frame of *ie1* is presented in capitals. The translation initiation codon (open box) and translation termination codon (asterisk) of ie1 are indicated. The 5 and 3 ends of *ie1* are indicated by arrow, and the pupative TATA (single underlined) and CAAT (double underlined) boxes are indicated. The differences among BmNPV-K1, AcNPV and BmNPV T3 sequences are indicated in boldface at nucleotide and amino acid sequence positions. Dashes indicate gaps introduced for optimal alignment. Identical sequences are indicated by dots below the AcNPV sequence. The sequence of BmNPV-K1 has been deposited in GenBank (Accession number AY048770).

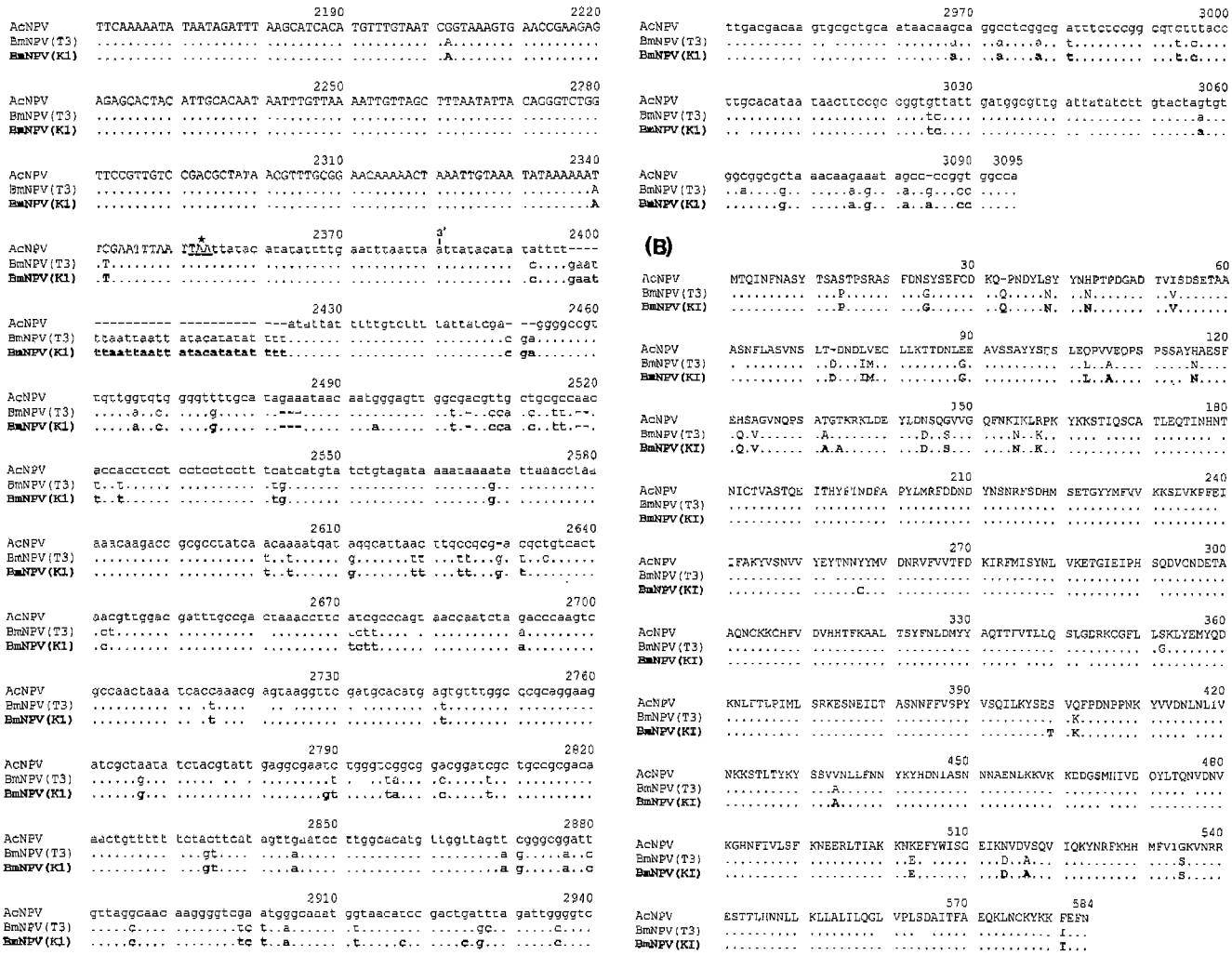

Fig. 2. Continued.

wild-type BmNPV-infected Bm5 cells. A total of 1×10^6 cells per 35-mm-diameter dish was infected at a multiplicity of infection of 5 PFU per cell. Cells were collected at 3, 6, 12, 18, and 24 hrs postinfection (p.i.). Total cellular RNA was isolated using Total RNA extraction kit (Promega).

Northern blot analysis

Total cellular RNA (10 μg per lane) from 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 ie1 gene transcripts was a 3.1 kb BmNPV-K1 *ie1* gene radiolabeled with [α-^{32}P] dCTP (Amersham). The other procedures for washing the membrane filter and exposing *X*-ray film were performed as in the Southern blot analysis described above.

Results and Discussion

The genome of AcNPV and BmNPV T3 was sequenced and analyzed (Ayres *et al.*, 1994; Gomi *et al.*, 1999). 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 at nucleotide level and about 93% at amino acid level. To identify *ie1* gene in BmNPV-K1, therefore, we have employed PCR based on the conserved region of *ie1* of AcNPV and BmNPV T3 so far known (Fig. 1A). The amplified PCR product, as expected, was observed 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 was cloned and then sequenced.

The nucleotide sequence of PCR product was analyzed and its amino acid was deduced. As the result of the complete nucleotide sequence (GenBank accession number; AY048770) in Fig. 2, the *ie1* gene has an open reading frame of 584 amino acids with a predicted MW of about 67 kDa. The 5 end contained the perfectly conserved tetra-nucleotide CAGT motif corresponding to the consensus transcription initiation sequence shared by other early bacu- lovirus genes (Blissard and Rohrman, 1990) as well as putative CAAT and TATA-like sequences GCATAAT and TATAAATT which are correctly conserved when compared with AcNPV and BmNPV T3 *ie1* (Guarino and Summers, 1987; Huybrechts *et al.*, 1992; Ribeiro *et al.*, 1994). A pupative TATA box is located 25 to 32 bp upstream of the cap site. A sequence similar to the consensus CAAT box is located 69 to 77 bp upstream of the start site for transcription. It was found that the 3 end in BmNPV T3 and AcNPV is located 52 bp and 31 bp downstream of the translation termination codon, respectively (Guarino and Summers, 1987; Huybreths *et al.*, 1992; Ribeiro *et al.*, 1994).

The nucleotide and deduced amino acid sequences of BmNPV-K1 *ie1* were compared with those of AcNPV and BmNPV T3 (Table 1 and 2). The nucleotide and amino acid sequences of the *ie1* coding region of BmNPV-K1 has 96% and 95% sequence identity to those of AcNPV, respectively. When the BmNPV-K1 *ie1* coding region and BmNPV T3 *ie1* are aligned, nucleotide and amino acids sequence homologies of 99.7% and 99.3% are respectively observed. The sequences of BmNPV-K1 *ie1* showed high homology with AcNPV and BmNPV T3 strain so far known (Guarino and Summers, 1987; Huybrechts *et al.*, 1992; Ribeiro *et al.*, 1994). The nucleotide sequences of BmNPV-K1 *ie1* were different from 6 positions in BmNPV T3. In addition, BmNPV-K1 *ie1* were different from amino acid sequence at 4 positions, 134, 257, 352 and 400, in BmNPV T3.

The localization of *ie1* gene in the BmNPV-K1 genome was confirmed by using Southern blot analysis. BmNPV-K1 genome was digested with *Cla*I and *Eco*RI restriction enzymes without restriction site within *ie1* gene, and hybridized with the full-length *ie1* gene amplified by PCR in this study as a probe (Fig. 3). The Southern blot analysis revealed that the *ie1* gene in BmNPV-K1 genome was localized on the 7.2 kb *Cla*I fragment and 9.8 kb *Eco*RI fragment.

Table 1. Alignment of the nucleotide sequence of the *ie1* coding region from BmNPV-K1

	1	2	3
AcNPV	-	4.62	5.13
BmNPV T3	27	-	0.68
BmNPV-K1	30	4	-

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 *ie1* coding region from BmNPV-K1

	1	2	3
AcNPV	-	3.70	3.76
BmNPV T3	65	-	0.34
BmNPV-K1	66	6	-

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

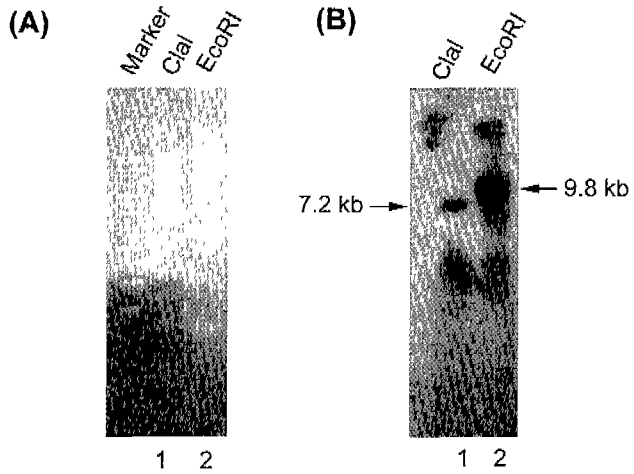

Fig. 3. Southern blot analysis of BmNPV-K1 genome. Viral DNAs digested with *Cla*I (lane 1) and *Eco*RI (lane 2) were electrophoresed through a 1.0% agarose gel (A), and hybridized at 42°C with a labeled probe (B). The probe used to detect DNA fragment containing *ie1* was a 3.1 kbp BmNPV-K1 *ie1* amplified by PCR in this study. Hybridized bands are indicated by arrow with molecular size.

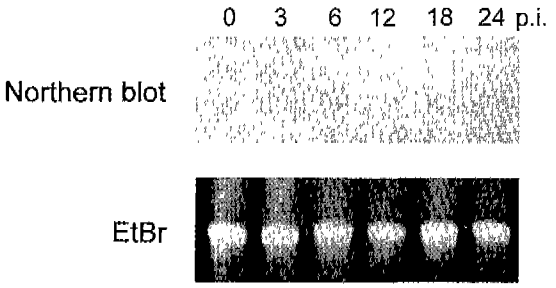

Fig. 4. Northern blot analysis of ie1 transcripts from BmNPV-K1-infected cells. Total RNA was collected from Bm5 cells at various times p.i. as indicated at the top of each lane. The probe used to detect *ie1* transcripts was a 3.1 kbp BmNPV-K1 *ie1* amplified by PCR in this study.

To verify whether the *ie1* transcripts were correlated with virus replication, we examined Northern blot analysis with *ie1* probe (Fig. 4). Total cellular RNA purified from Bm5 cells 3, 6, 12, 18, and 24 hrs p.i. with wild-type BmNPV-K1 was hybridized with an excess of probe. As shown in Fig. 4, *ie1* transcripts were detected at 3 hrs p.i. and maximally observed at 6 hrs p.i. These *ie1* transcripts were maintained during 24 hrs p.i., but slightly decreased at 12 hrs p.i. Thus, this result was consistent with previous results that levels of *ie1* message were maintained during the first 24 hrs of infection and IE1 accumulated from 4 to 72 hrs p.i. (Choi and Guarino, 1995; Guarino and Summers, 1987).

In conclusion, we report gene structure of the BmNPV-K1 *ie1* gene for the first time. This gene contained the same or similar features typical to the most *ie1* genes reported previously. Knowledge of the *ie1* gene in this study will provide the information for establishing Bm NPV-K1 strain. The BmNPV-K1 *ie1* will provide a means of developing transformed *B. mori* cell lines.

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