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Molecular Cloning and Characterization of Very Late Expression Factor 1 Gene, *vlf-1* from *Bombyx mori* Nuclear Polyhedrosis Virus K1

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We cloned and characterized a very late expression factor 1 gene, *vlf-1*, which regulates the level of very late gene transcripts, from *Bombyx mori* nuclear polyhedrosis virus (BmNPV) K1 strain. The 1,140 bp *vlf-1* has an open reading frame of 379 amino acid and a MW of 44 kDa. The *vlf-1* nucleotide sequence of BmNPV-K1 showed high homology with *Autographa californica* nuclear polyhedrosis virus and BmNPV T3 strain so far known, and its deduced amino acid residues were identical to those of BmNPV T3. The location of *vlf-1* in the BmNPV-K1 genome was confirmed by Southern blot analysis and its expression patterns at the transcriptional level were confirmed by Northern hybridization analysis.

Key words : Baculovirus, *Bombyx mori* nuclear polyhedrosis virus, Very late expression factor 1 gene (*vlf-1*)

Introduction

Baculoviruses possess a large circular DNA genome which replicates in the nuclei of infected cells and is transcribed in three temporally distinct phases: early, late, and very late. The promoters of the genes encoding the polyhedrin and p10 proteins of baculovirus are most frequently employed in baculovirus expression vector systems to express heterologous gene (King and Possee, 1992; OReilly *et al.*, 1992). Both promoters are strongly activated during the very late stage of infection, which are activated at between 18 and 24 hrs postinfection (p.i.).

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Late gene transcription is mediated by a novel, α-amanitin-resistant RNA polymerase activity which is induced during virus infection (Glocker *et al.*, 1993; Grula *et al.*, 1981; Huh and Weaver, 1990a, 1990b) and is probably encoded, at least in part, by the viral genome (Passarelli *et al.*, 1994). Very late gene expression, which is required for occluded virus formation, is also mediated by an α-amanitin-resistant RNA polymerase but additionally requires the function of a novel gene, very late expression factor 1 gene (*vlf-1*), which is predicted to encode a polypeptide with sequence motifs characteristic of a family of integrase/resolvases (McLachlin and Miller, 1994). The promoters of most late and very late genes have novel properties, including an absolute dependence on a TAAG sequence located at the initiation point of transcription (Morris and Miller, 1994; Ooi *et al.*, 1989).

The *vlf-1* previously identified by analysis of a temperature-sensitive mutant of *Autographa californica* nuclear polyhedrosis virus (AcNPV) (McLachlin and Miller, 1994) in the transient-expression assay and found that *vlf-1* specially transactivated the very late promoters and VLF-1 is the primary regulator of very late gene expression (Todd *et al.*, 1996). Thus, the *vlf-1* is required for strong expression of the polyhedrin gene and is expressed primarily as a late gene. By altering the level and/or timing of *vlf-1* expression, the timing of polyhedrin gene (*polh*) expression, which normally occurs very late in infection, could be advanced or delayed (Yang and Miller, 1998a). Early overexpression of *vlf-1* increased the level of expression from the *polh* promoter. Because expression of *polh* responds to expression of *vlf-1*, VLF-1 can provide a means of regulating baculovirus expression vector systems employing the *polh* promoter to drive foreign gene expression (Yang and Miller, 1998b).

In this study, we have cloned and characterized *vlf-1* from *Bombyx mori* nuclear polyhedrosis virus K1 strain

(BmNPV-K1) (Kang *et al.*, 1997). The sequence of BmNPV-K1 *vlf-1* was aligned to that of AcNPV (McLachlin and Miller, 1994) and BmNPV T3 (Gomi *et al.*, 1999).

Materials and Methods

Cells and virus

The *Spodoptera frugiperda* IPLB Sf21-AE (Vaughn *et al.*, 1977) clone 9 (Sf9) and *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 AcNPV (Lee and Miller, 1978) was propagated in Sf9 cells. Wild-type BmNPV-K1 (Kang *et al.*, 1997) and BmNPV T3 (Gomi *et al.*, 1999) were propagated and titered in Bm5 cells. The titer was expressed as plaque forming units (PFU) per ml (OReilly *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 *vlf-1* was amplified from viral DNAs using the primer 5'-GATAGTATTGACACCGATTCTCC-3' and 5'-CCCTTACTCTATTCGTTGCG-3', annealing to the 5' promoter region and 3' untranslational region respectively (McLachlin and Miller, 1994). After 35-cycle amplification (94°C for 1 min; 55°C for 1 min; 72°C for 1 min), PCR products were ethanol precipitated, centrifuged at 10,000 × g for 15 min, and rinsed with 70% ethanol. These DNAs were analyzed by 1% agarose gel electrophoresis. The PCR products for sequencing were cloned into pGem-T vector (Promega).

DNA sequencing

By utilizing double-stranded DNA templates synthesized by PCR, both strands were sequenced across the entire region by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

Multiple sequence alignment

Protein sequence homology searches were performed by using the predicted amino acid sequence of VLF-1 (accession number S36692; AcMNPV hypothetical protein ORF 1137) and the basic local alignment search tool (BLAST) (Altschul *et al.*, 1990) to search the National Center for Biotechnology Information nonredundant peptide sequence database. Sequence alignments were conducted by using the Pileup multiple sequence alignment program of the Genetics Computer group (Madison) sequence analysis software package. The following list includes the accession numbers for the sequences used in the multiple sequence alignments; the sequences were derived from either the GenBank or Swiss-Prot database: VLF-1 AcMNPV (S36692); *vlf-1* AcMNPV (L22858); and *vlf-1* BmNPV T3 (L33180).

Southern blot analysis

Viral DNAs digested with *Eco*RV and *Sal*I were electrophoresed through 1.0% agarose gel as described previously (O'Reilly *et al.*, 1992). The DNA of the gel was transferred onto a nylon blotting membrane (Schleicher & Schuell) and hybridized at 42°C. The probe used to detect DNA fragment containing *vlf-1* was a 1140 bp BmNPV-K1 *vlf-1* amplified by PCR in this study.

RNA isolation

Total cellular RNA was isolated from mock-infected or 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 4, 8, 12, 18, 24, and 48 hrs p.i. Total cellular RNA was isolated by use of a guanidinium isothiocyanate procedure

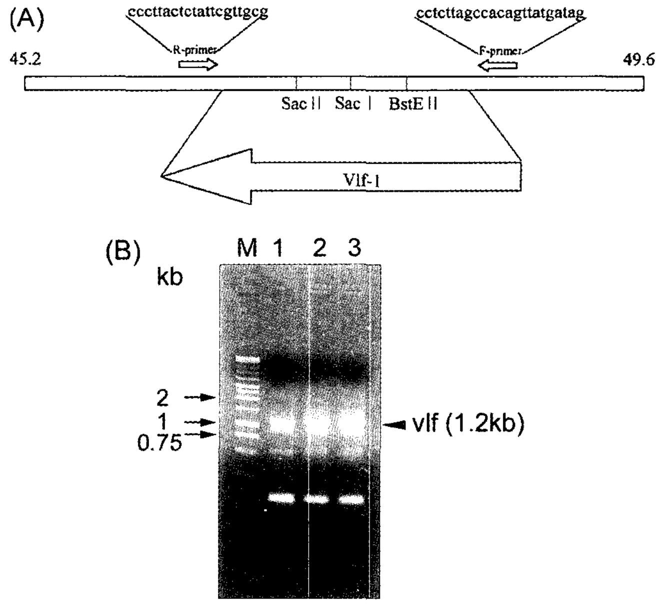

Fig. 1. PCR of *vlf-1* from the genome of baculoviruses. The PCR primers for identification of BmNPV-K1 *vlf-1* were based on the previously identified *vlf-1* within 45.2 to 49.6 map units of AcMNPV (A), as described in Materials and Methods. The amplified PCR products were analyzed by 1% agarose gel electrophoresis (B). AcNPV (lane 1), BmNPV T3 (lane 2), Bm NPV-K1 (lane 3), and DNA size markers (M) are indicated.

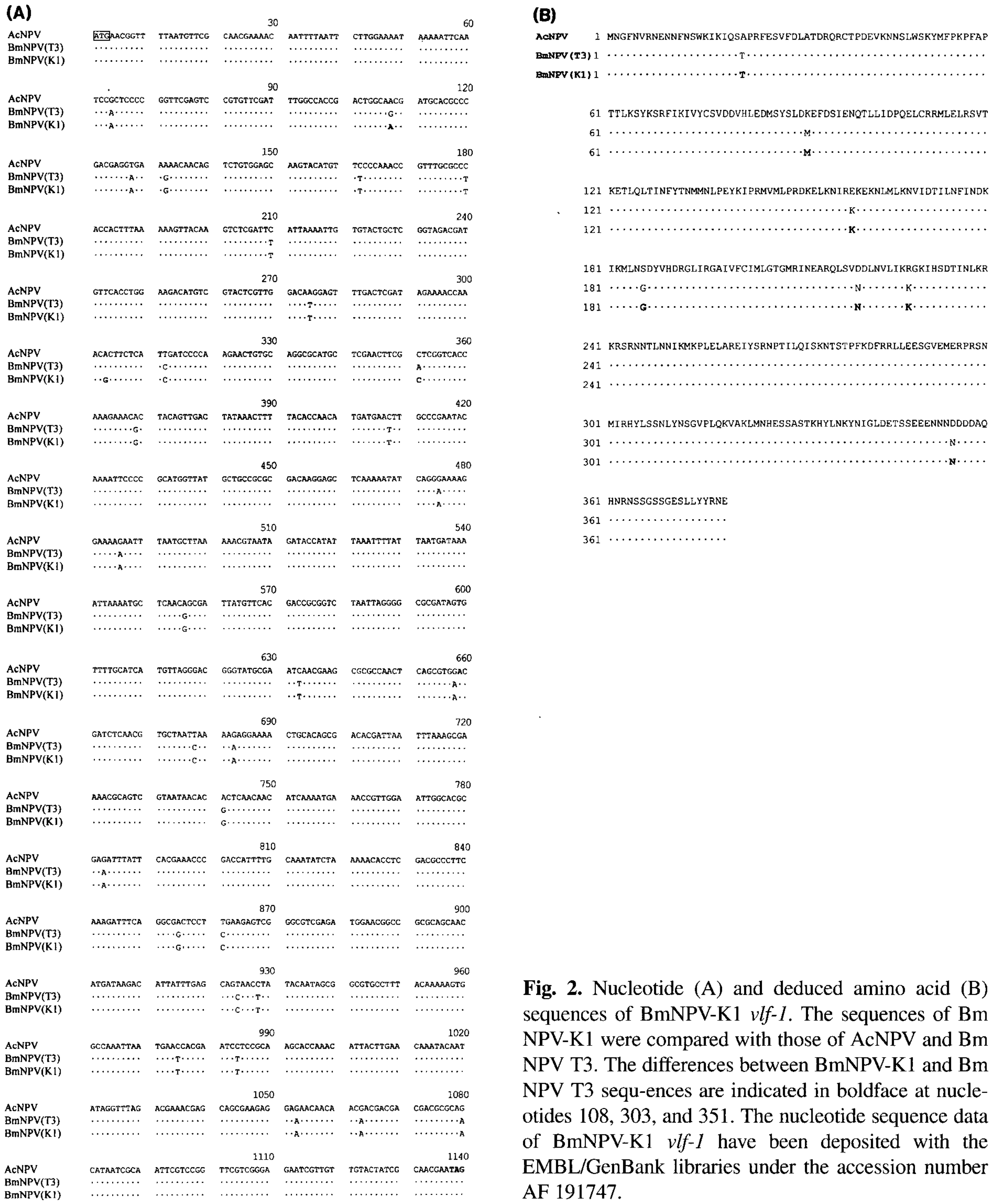

Fig. 2. Nucleotide (A) and deduced amino acid (B) sequences of BmNPV-K1 *vlf-1*. The sequences of Bm NPV-K1 were compared with those of AcNPV and Bm NPV T3. The differences between BmNPV-K1 and Bm NPV T3 sequ-ences are indicated in boldface at nucleotides 108, 303, and 351. The nucleotide sequence data of BmNPV-K1 *vlf-1* have been deposited with the EMBL/GenBank libraries under the accession number AF 191747.

(Chirgwin *et al.*, 1979).

Dot blot analysis
Total cellular RNA (1 μg per well) from infected cells was denatured by glyoxalation (McMaster and Carmichael, 1977), transferred onto a nylon blotting membrane (Schleicher & Schuell) and hybridized at 42°C in the presence of 50% formamide. The probe used to detect *vlf-1* transcripts was a 1140 bp BmNPV-K1 *vlf-1* amplified by PCR in this study.

Nucleotide sequence accession number
The sequence data ob'tained from this study have been deposited with the EMBL/GenBank libraries under the

accession number AF191747.

Results and Discussion

To identify *vlf-1* in BmNPV-K1, we have designed the PCR primer set based on the sequences of the conserved region of *vlf-1* of AcNPV and BmNPV T3 so far known (Fig. 1A). The amplified PCR products, as expected, were observed in three baculoviruses (Fig. 1B). As shown in Fig. 1, the molecular size of the products in three baculoviruses was identical to that expected. The PCR products for sequencing were cloned (data not shown).

The nucleotide sequence of PCR products was analyzed and its amino acid was deduced. As the result of the complete nucleotide sequence (GenBank accession number; AF191747) in Fig. 2, the 1,140 bp *vlf-1* has an open reading frame of 379 amino acid and a predicted MW of 44 kDa. The nucleotide and deduced amino acid sequences were compared with those of AcNPV and BmNPV T3. The sequences of BmNPV-K1 showed high homology with AcNPV and BmNPV T3 strain so far known (McLachlin and Miller, 1994; Gomi *et al.*, 1999). The *vlf-1* of BmNPV-K1 was different from nucleotide sequences at positions, 108, 303 and 351 in BmNPV T3. However, deduced amino acid sequences of *vlf-1* of BmNPV-K1 were identical to those of BmNPV T3.

The localization of *vlf-1* in the BmNPV-K1 genome was confirmed by using Southern blot analysis. BmNPV-K1 genome was digested with *Eco*RV and *Sal*I, and proved with amplified *vlf-1* (Fig. 3). The *vlf-1* in BmNPV-K1 genome was localized on the 2.8 kb *Eco*RV fragment and 13.8 kb *Sal*I fragment.

To verify whether the *vlf-1* transcripts were correlated with virus replication, we examined dot hybridization analysis with *vlf-1* probe (Fig. 4). As shown in Fig. 4, *vlf-1* transcripts were being dramatically transcribed in the wild-type BmNPV-K1-infected cells at 18 hr p.i. Thus, this result was consistent with the previous result that revealed a correlation between the timing of *vlf-1* expression and the timing and/or level of polyhedrin and p10 synthesis in the wild-type AcNPV-infected cells (Yang and Miller, 1998b). Actually, the fact that transcription from the *polh* and *p10* promoters is strongly activated at 18 and 24 hrs p.i was previously reported (King and Possee, 1992; O'Reilly *et al.*, 1992). In addition, the product of *vlf-1* is known to be involved in the regulation of polyhedrin synthesis at the transcriptional level as a limiting factor in very late gene expression (Yang and Miller, 1998b).

Knowledge of the *vlf-1* in this study will provide an information for establishing BmNPV-K1 strain. The *vlf-1* BmNPV-K1 will now provide a means of developing transformed *B. mori* cell line expressing *vlf-1*.

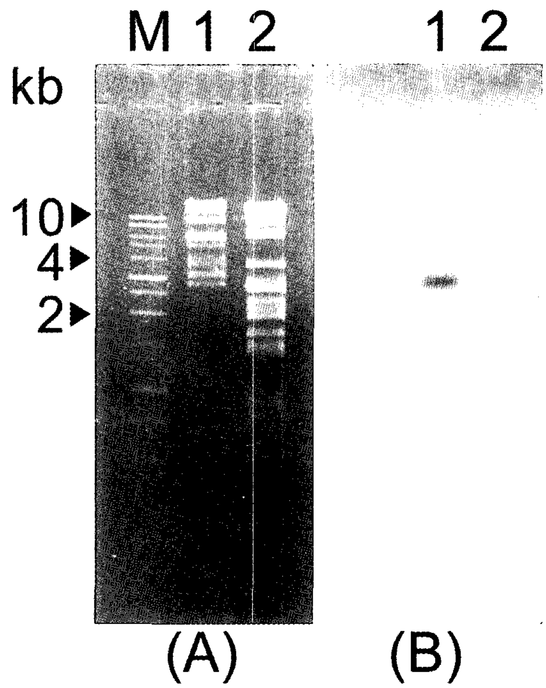

Fig. 3. Southern blot analysis of BmNPV-K1 genome. Viral DNAs digested with *Eco*RV (lane 1) and *Sal*I (lane 2) were electrophoresed through a 1.0% agarose gel (A) and hybridized at 42°C with a labeled probe (B). The probe used to detect DNA fragment containing *vlf-1* was a 1140 bp BmNPV-K1 *vlf-1* amplified by PCR in this study. The DNA size markers (M) are indicated in kilobases.

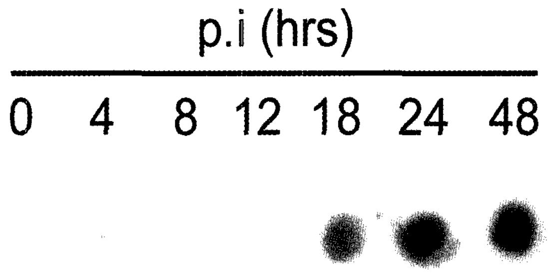

Fig. 4. Dot blot analysis of *vlf-1* transcripts from BmNPV-K1-infected cells. Total cellular RNA was collected from Bm5 cells at various times p.i. as indicated at the top of each well. The probe used to detect *vlf-1* transcripts was a 1140 bp BmNPV-K1 *vlf-1* amplified by PCR in this study.

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