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Construction and Characterization of Transformed Insect Cells Expressing Baculovirus Very Late Factor in an Infection-Independent Manner

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Transformed *Spodoptera frugiperda* (Sf9) cells expressing baculovirus very late factor (VLF-1) were constructed by using *Autographa californica* nuclear polyhedrosis virus (AcNPV) immediate early gene (*ie1*). Neomycin-resistance gene as a selectable marker was introduced under the control of AcNPV *ie1* promoter, and *Bombyx mori* nuclear polyhedrosis (BmNPV-K1) *vlf-1* gene was introduced under the control of the *Drosophila* heat shock protein gene (*hsp70*) promoter to yield dual expression plasmid with two independent transcription units. It was transfected into Sf9 cells and cell clones expressing vlf-1 were selected by G418 treatment. Genomic DNA from transformed cells was isolated and integration of AcNPV *ie1* harboring *vlf-1* was confirmed by PCR using AcNPV *ie1*-specific primers and Southern blot analysis. The transformed cells expressing VLF-1 in an infection-independent manner expressed foreign gene product of recombinant baculovirus in the earlier stage of infection compared with control Sf9 cells. These results suggest the possible to develop highly efficient transformed insect cells for baculovirus expression vector system.

Key words : Baculovirus, Transformed insect cells, Very late expression factor 1 gene (*vlf-1*), Recombinant virus

Introduction

Baculoviruses are used as vectors for the high-level expression of the foreign gene in insect cells. The baculovirus expression vector system usually utilizes the strong promoters of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) or *Bombyx mori* nuclear polyhedrosis virus (BmNPV). 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; O'Reilly *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.).

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. Among these early genes of baculovirus, the immediate early 1 gene (*ie1*) of AcNPV has been studied extensively. This gene is expressed immediately upon entrance of the viral genome into the host cell, even in the absence of concomitant protein synthesis (Guarino and Summers, 1987). The transformed lepidopteran cells (Sf9) were produced by the AcNPV *ie1* promoter, and expressed a foreign gene product continuously in the absence of viral infection (Jarvis *et al.*, 1990). The generation of transformed lepidopteran cells expressing foreign proteins continuously has also been reported (Jarvis *et al.*, 1990; Joyce *et al.*, 1993).

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

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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* is 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 is 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* increases 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). To advance polyhedrin synthesis, moreover, the *vlf-1*-expressing cell line has been developed that match with recombinant baculovirus specialized by some engineering (Yang and Miller, 1998b). In addition, the *vlf-1* genes of AcNPV and BmNPV are known to share high homology in amino acid sequences (McLachlin and Miller, 1994; Gomi *et al.*, 1999; Park *et al.*, 2000).

In this study, therefore, we have constructed an insect cell line that expresses BmNPV-K1 VLF-1 under the control of the *Drosophila* heat shock protein gene (*hsp70*) promoter in an infection-dependent manner. The transformed insect Sf9 cells expressed foreign genes at earlier stages of general recombinant baculovirus infection by expressing VLF-1 continuously. We have also expressed βgalactosidase from recombinant baculovirus in the transformed Sf9 cells, and compared the expression levels in transformed cells to those in a control cells.

Materials and Methods

Cells and recombinant virus

The *Spodoptera frugiperda* IPLB Sf21-AE (Vaughn *et al.*, 1977) clone 9 (Sf9) cells and transformed cells were grown at 27°C in TC100 medium (GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL) (O'Reilly *et al.*, 1992). Recombinant AcNPV (BacPAK 6, Clontech) was propagated in Sf9 cells. The titer was expressed as plaque forming units (PFU) per ml (O'Reilly *et al.*, 1992).

Construction of expression plasmid

The pAcIE1-neo (Cho *et al.*, 1998), pAcIE1-del (Cho *et al.*, 1998), and pHsSV (Kang, 1996) were described previously. Plasmid pAcIE1-neo-Hsp was constructed by insertion of both *ie1* promoter and neomycin-resistance gene from pAcIE1-neo into *Kpn*I site of the front of *hsp70* promoter of pHsSV following *ie1* sequence from pAcIE1-del into *Bam*HI site behind SV40 poly(A+) tail of pAcIE1p-neo-Hsp. Plasmid pAcIE1-neo-Hsp-vlf was constructed by digestion of pAcIE1-neo-Hsp with *Hin-d*III, blunt-ending and ligation with BmNPV-K1 *vlf-1*.

Transfection

Cell culture dish (35-mm diameter) was seeded with 1×10^9 Sf9 cells and incubated at 27°C for 1 hr to allow the cells to attach. Five micrograms of pAcIE1-neo-Hsp-vlf plasmid DNA in 20 mM HEPES buffer and sterile water to make a total volume of 50 μl were mixed in a polystyrene tube. Fifty microliters of 100 μg/ml Lipofectin (GIBCO/BRL) were gently mixed with the DNA solution, and the mixture was incubated at room temperature for 30 min. The cells were washed twice with serum-free TC100 medium. Serum-free TC100 (1.5 ml) was added to each dish. The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubation at 27°C for 5 hrs, 1.5 ml TC100 containing antibiotics and 10% FBS was added to each dish and the incubation at 27°C continued. At 3 days, cells were selected in TC100 medium containing 10% FBS and 1 mg/ml G418 (GIBCO/BRL) for two weeks. Individual clones of neomycin-resistant cells were picked and amplified. Cells were visualized by inverted phase contrast microscope (Nikon).

Genomic DNA extraction

The transformed cell clones were cultured in TC100 medium containing 10% FBS and 1 mg/ml G418. Genomic DNA was extracted from the cell pellets (1×10^6 cells) by using Wizard™ genomic DNA purification kit according to the manufacturers instructions (Promega).

Polymerase chain reaction (PCR)

Genomic DNAs from transformed or control Sf9 cells were used as templates. The PCR primer set was based on the sequences of the *vlf-1* and *ie1*. The primers were prepared to 5-CGACACAGATCAAGATGTCC-3 for the *vlf-1* gene and 5-CCTCTTCAGCCACAGTTAATGATAG-3 for the *ie1* gene. 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).

Southern blot analysis

Genomic DNAs from transformed or control Sf9 cells were digested with *Eco*RV or *Bam*HI and electrophoresed through 1.0% agarose gel. 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 as described previously (Park *et al.*, 2000).

RNA isolation

Total cellular RNA was isolated from mock-infected or recombinant virus-infected cells, transformed or control Sf9 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 6, 9, 12, 15, 18, and 24 hrs p.i. Total cellular RNA was isolated using Total RNA extraction kit (Promega).

Dot blot analysis

Total cellular RNA (1 μg per well) from transformed or control Sf9 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 (Park *et al.*, 2000). The probe used to detect β-galactosidase transcripts by recombinant virus infection in transformed or control Sf9 cells was a 3.2 kb *E. coli* βgalactosidase gene.

SDS-PAGE and Western blot analysis

Transformed or control Sf9 cells were mock infected or infected with recombinant AcNPV expressing β-galactosidase in a 35-mm diameter dish (1×10^6 cells) at a MOI of 5 PFU per cell. After incubation at 27°C, cells were harvested at 6, 9, 12, 15, 18, and 24 hrs p.i. For SDS-PAGE of cell lysates, cells infected with or without recombinant virus were washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) and mixed with a sample buffer (5% SDS, 10% β-mercaptoethanol, 0.02% bromophenol blue, 20% glycerol). Samples were boiled for 5 min and clarified by centrifugation (10,000 × g for 1 min). The total cellular lysates were subjected to 10% SDS-PAGE (Laemmli, 1970), electroblotted and incubated with β-galactosidase antibody (Clontech) (Towbin *et al.*, 1979).

β-Galactosidase activity assay

Transformed or control Sf9 cells were mock infected or infected with recombinant AcNPV expressing β-galactosidase in a 35-mm diameter dish (1×10^6 cells) at a MOI of 5 PFU per cell. After incubation at 27°C, cells were harvested at 6, 9, 12, 15, 18, and 24 hrs p.i. The culture supernatants were clarified by centrifugation (10,000 × g for 1 min) and transferred in 100 μl portions to a 96-well microplate. β-Galactosidase activity of these samples was assayed using β-Galactosidase assay kit according to the manufacturers instructions (Promega).

Results

The expression plasmid used in this study is depicted in Fig. 1. Plasmid pAcIE1-neo-Hsp was constructed in which a neomycin-resistance gene as a selectable marker was placed under the control of the *ie1* promoter and then *hsp70* promoter was introduced behind the neomycin-resistance gene. To facilitate the construction and further manipulation of expression plasmid containing foreign genes, cloning site for foreign genes was inserted in the *Hind*III site under the control of *hsp70* promoter. SV40 poly (A+) site was used for transcription termination followed *ie1* sequence for integration into genome of Sf9 cells. This intermediate plasmid pAcIE1-neo-Hsp is a generally useful vector for the transformation of insect cells (Fig. 1A). The *vlf-1* gene from BmNPV-K1 was then cloned into this *Hind*III site, yielding pAcIE1-neo-Hsp-vlf (Fig. 1B). The expression vector pAcIE1-neo-Hsp-vlf has two independent transcription units in same orientation.

Sf9 cells were transfected with the expression plasmid pAcIE1-neo-Hsp-vlf and the cell clones surviving in the presence of G418 were measured for 2 weeks post-transfection. Significant numbers of G418-resistant survivors were observed after transfection with pAcIE1-neo-Hsp-vlf, but not in the control Sf9 cells (Fig. 2). G418-resistant cells were selected and clones were picked. The selected clone was amplified for the further characterization.

To identify *vlf-1* in the selected cell clone, genomic DNA was isolated from control Sf9 cells and transformant. We have designed the PCR primer set based on the sequences of the *vlf-1* and *ie1* (Fig. 3A). The amplified PCR products, as expected, were observed in transformant, but not in the control Sf9 cells (Fig. 3B). As shown in Fig. 3, the molecular size of the PCR products in transformant was identical to that expected. Partial nucleotide sequencing and restriction enzyme digestion (data not

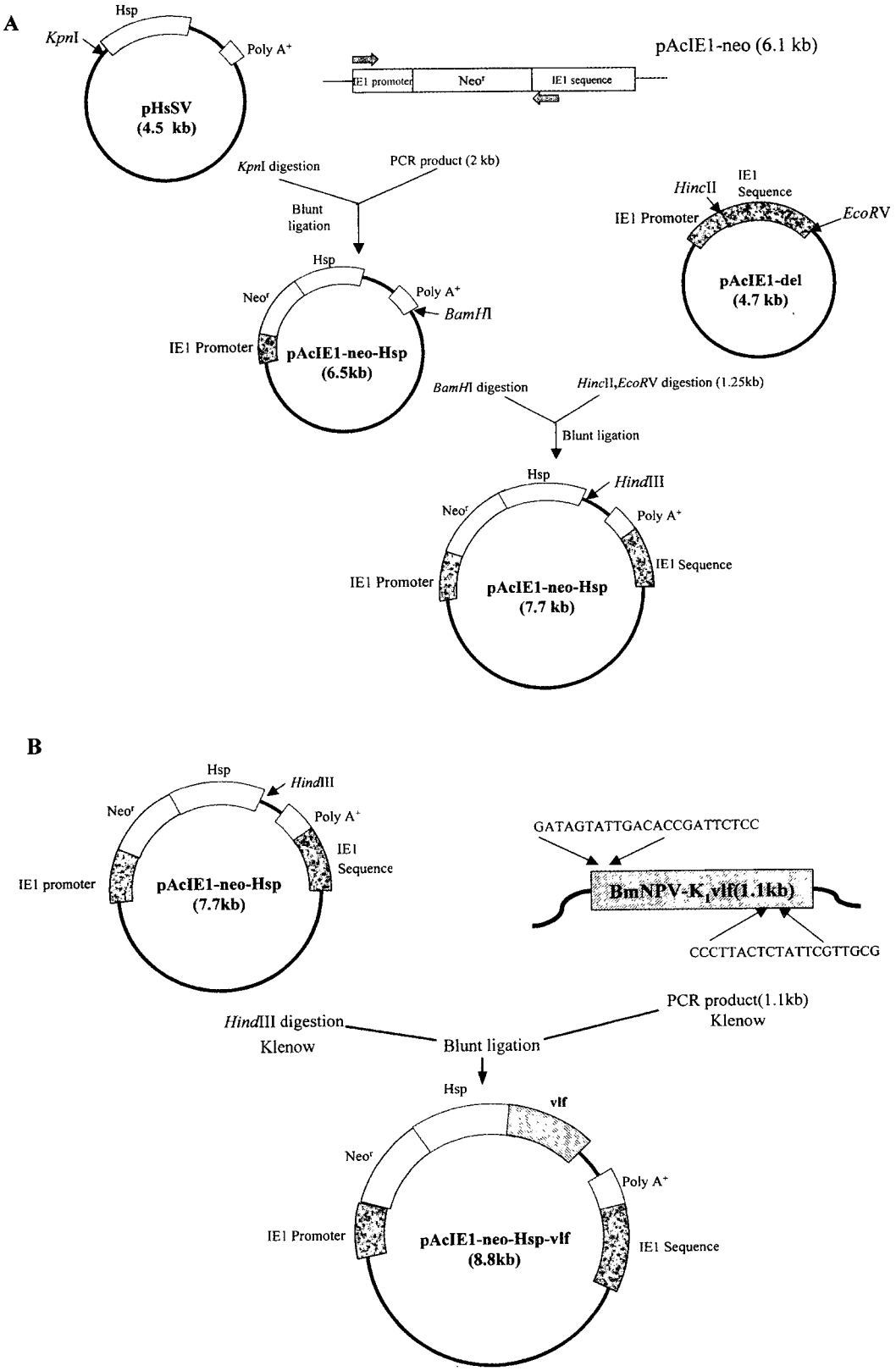

Fig. 1. Construction of expression plasmid. (A) Plasmid pAcIE1-neo-Hsp contains *ie1* promoter, neomycin-resistance gene (Neo^r), *hsp70* promoter (Hsp), cloning site (*Hind*III), and SV40 polyadenylation signals (poly A+). (B) Plasmid pAcIE1-neo-Hsp-vlf was constructed by insertion of BmNPV-K1 *vlf-1* (vlf) into *Hind*III cloning site of plasmid pAcIE1-neo-Hsp.

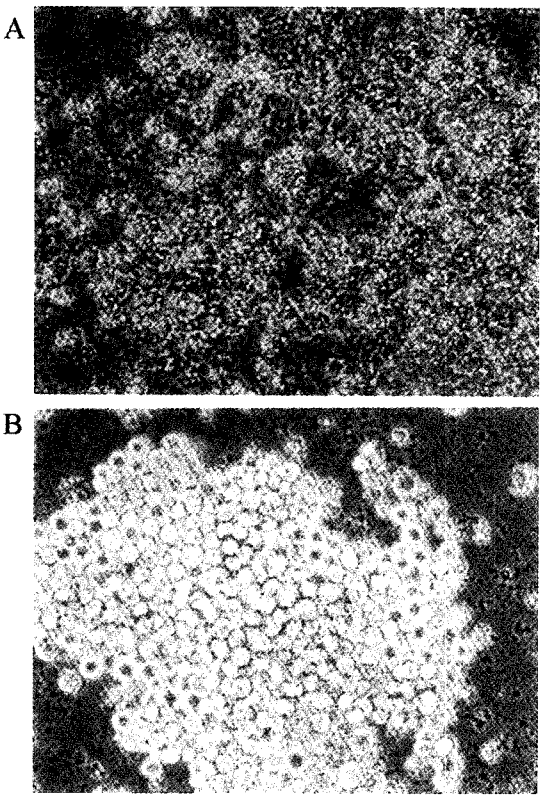

Fig. 2. Micrographs of the transformed Sf9 cells. Control (A) and transformed (B) Sf9 cells were cultured in the presence of G418. Transformed Sf9 cells were survived in the presence of G418.

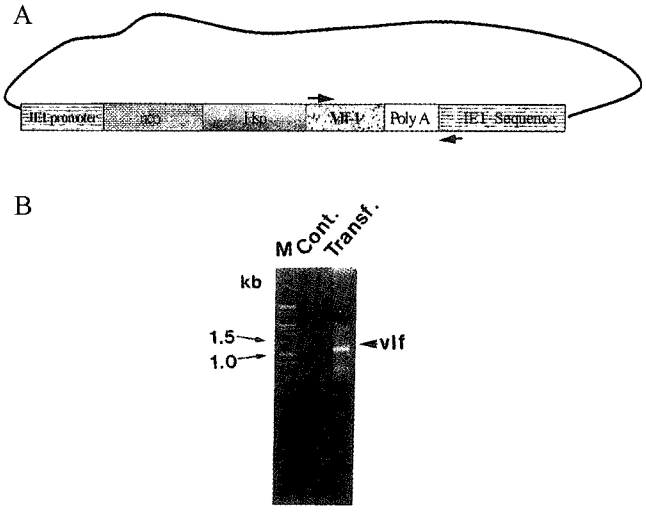

Fig. 3. PCR of the genomic DNA from transformed cells. The PCR primers for identification of BmNPV-K1 *vlf-1* and *ie1* sequences from the genomic DNA of transformed cells were based on the expression plasmid pAcIE1-neo-Hsp-vlf (A). The amplified PCR products were analyzed by 1% agarose gel electrophoresis (B). DNA size markers, control Sf9 cells and transformed Sf9 cells are indicated at the top of each lane.

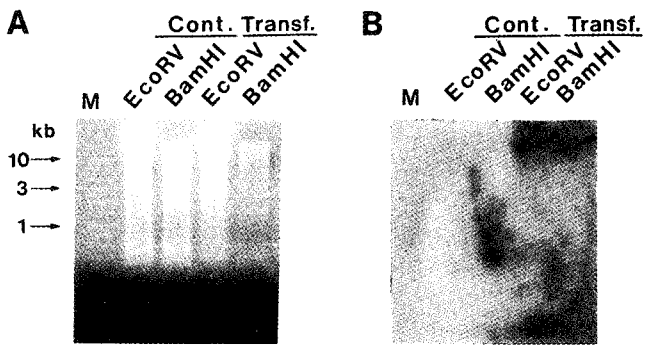

Fig. 4. Southern blot analysis of the genomic DNA from transformed cells. Genomic DNAs digested with *Eco*RV or *Bam*HI were electrophoresed through a 1.0% agarose gel (A) and hybridized at 42°C with a labeled probe (B). The probe used to detect DNA fragment containing *vlf-1* was a 1140 bp BmNPV-K1 *vlf-1* amplified by PCR. DNA size markers, restriction enzymes, control Sf9 cells and transformed Sf9 cells are indicated at the top of each lane.

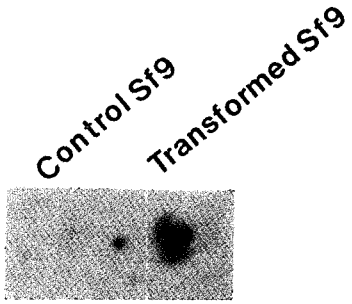

Fig. 5. Dot blot analysis of *vlf-1* transcripts from transformed Sf9 cells. Total cellular RNA was isolated from transformed Sf9 cells at 30 passages. The probe used to detect *vlf-1* transcripts was a 1140 bp BmNPV-K1 *vlf-1* amplified by PCR. Control and transformed Sf9 cells are indicated at the top of each well.

shown) confirmed the result obtained from PCT analysis. Furthermore, genomic DNA isolated from control Sf9 cells and transformant was digested with *Eco*RV or *Bam*HI, and Southern blot analysis was probed for *vlf-1*. The probe hybridized with single DNA band digested with *Eco*RV or *Bam*HI from transformant, but not in the control Sf9 cells (Fig. 4).

Dot blot analysis was performed to determine *vlf-1* transcription in the transformant at passages 30 (Fig. 5). This showed that the *vlf-1* gene was transcribed under the control of *hsp70* promoter, as expected from the ability of the transformant to survive in the presence of G418. Dot blot pattern from the transformant, but not in the control Sf9 cells, showed maintenance for at least 30 passages in this transformed cells.

To assess whether the expected expression of foreign

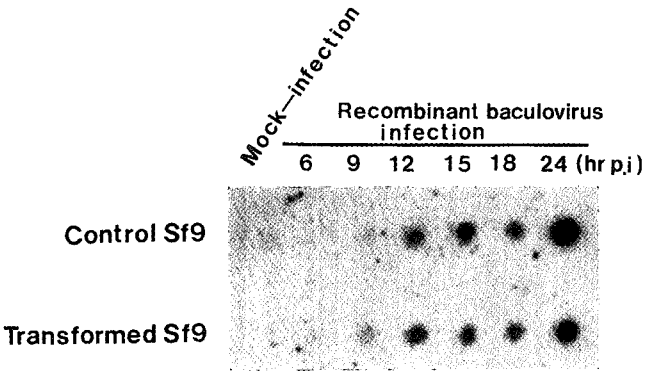

Fig. 6. Dot blot analysis of β-galactosidase transcripts from control or transformed Sf9 cells infected with recombinant baculovirus. Total cellular RNA was isolated from control or transformed Sf9 cells infected with recombinant AcNPV expressing βgalactosidase at 6, 9, 12, 15, 18, and 24 hrs p.i. The probe used to detect β-galactosidase transcripts was a 3.2 kb *E. coli* βgalactosidase gene. Control and transformed Sf9 cells are indicated at the left of panel and time post-infection is indicated at the top of each well.

gene product by recombinant baculovirus multiplication in the transformed cells is being accomplished, recombinant baculovirus expressing βgalactosidase was infected into transformed Sf9 cells. RNA was isolated from infected cells and this dot blot was probed for βgalactosidase gene (Fig. 6). The difference between the levels of βgalactosidase transcripts observed in transformed and control Sf9 cells was not as great as that expected. However, the dot blot patterns showed quantitative difference at early stage of infection. The β-galactosidase transcripts in transformed cells were found in trace amounts as early as 6 hrs p.i. Clearly, β-galactosidase transcripts were detected at 6 to 9 hrs p.i . in transformed cells, at least 3 hrs earlier than the control Sf9 cells.

We also assessed the effect of transformed cells harboring *vlf-1* on foreign gene expression in SDS-PAGE and Western blot analyses (Fig. 7). As expected, in transformed cells, β-galactosidase expression detected more rapidly than in the control cells. The β-galactosidase activity assay also revealed that transformed cells were more highly expressed at earlier stage of infection than the control Sf9 cells (Fig. 8). At 24 hrs p.i., β-galactosidase expression in transformed cells increased about 15%. Consistent with results of Fig. 7, transformed cells expressing VLF-1 were influenced primarily in the timing of expression from the *polh* promoter.

Discussion

We have constructed and characterized the transformed

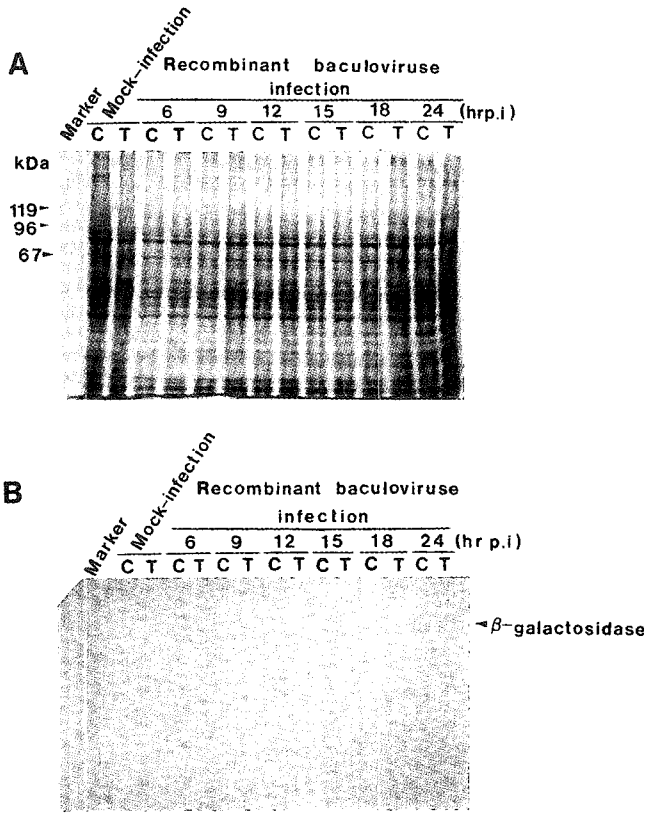

Fig. 7. SDS-PAGE (A) and Western blot analysis (B) of control or transformed Sf9 cells infected with recombinant baculovirus. Control or transformed Sf9 cells were mock infected or infected with recombinant AcNPV expressing βgalactosidase. The cells were harvested at 6, 9, 12, 15, 18, and 24 hrs p.i. The total cellular lysates were subjected to 10% SDS-PAGE, electroblotted and incubated with βgalactosidase antibody. Control (C) and transformed (T) Sf9 cells and time post-infection are indicated at the top of each lane. βGalactosidase expressed is marked on the right.

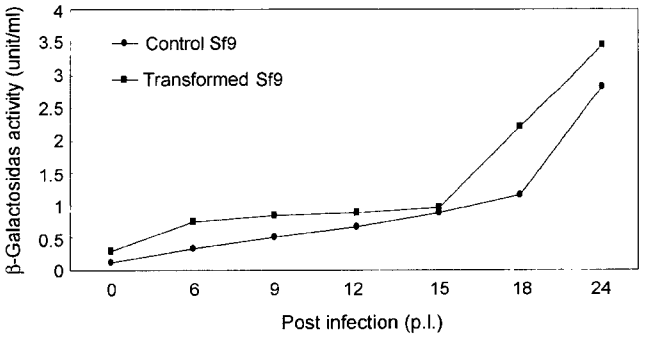

Fig. 8. The activity assay of βgalactosidase expressed from control or transformed Sf9 cells infected with recombinant baculovirus. Control (closed circle) or transformed (closed square) Sf9 cells were infected with recombinant AcNPV expressing β-galactosidase and harvested at 6, 9, 12, 15, 18, and 24 hrs p.i.

insect Sf9 cells expressing VLF-1 continuously. Expression plasmid, pAcIE1-neo-Hsp, containing *Hind*III cloning site for foreign genes could be utilized as generally useful vector in insect cells. The plasmid was based on the AcNPV *ie1* and *Drosophila hsp70* promoters (Jarvis *et al.*, 1990; Toeroek and Karch, 1980; Clem and Miller, 1994; Mori *et al.*, 1995). Previously, the *ie1* promoter was transcriptionally active in the absence of *de novo* viral protein synthesis in Sf9 cells (Guarino and Summers, 1986) and the generation of transformed lepidopteran cells expressing foreign proteins continuously has also been demonstrated (Jarvis *et al.*, 1990; Joyce *et al.*, 1993). For the easy selection of transformed cells, finally, dual expression vector pAcIE1-neo-Hsp-vlf was constructed, each initiating transcription of either the neomycin-resistance gene or BmNPV-K1 *vlf-1* with two independent transcription units in same orientation.

We have constructed an insect cell line that expresses VLF-1 under the control of the *hsp70* promoter in an infection-dependent manner. A cell line expressing VLF-1 was previously described in a study of *vlf-1* control of polyhedrin gene expression (Yang and Miller, 1998b). In the study, *vlf-1*-expressing cell line have been developed, which matches with recombinant baculovirus specialized by some engineering. The cell line reported here differs from the previous report in an important way. A cell line constructed in this study can be matched to general recombinant baculovirus.

In this study, foreign gene product of recombinant baculovirus was detected at 6 to 9 hrs p.i . in transformed cells, at least 3 hrs earlier than the control Sf9 cells. At 24 hrs p.i., furthermore, foreign gene expression in transformed cells increased about 15%. Thus, the influence of transformed cells expressing VLF-1 is manifested primarily in the timing of expression from the *polh* promoter. The increased expression level in transformed cells is clearly stemmed from the activation of *polh* promoter of BmNPV-K1 VLF-1, which shows high homology with AcNPV VLF-1 (Park *et al.*, 2000). The overall level of foreign gene product by recombinant baculovirus multiplication in transformed cells can be increased at earlier stage of infection than the control Sf9 cells, but the benefit is minor than expected. This was demonstrated in pAcIE1 -neo-Hsp-vlf, which produces VLF-1 to support foreign gene expression at the earlier stage of recombinant baculovirus infection but not enough to activate very late gene *polh* promoter as expected. One of the possible reasons for this observation could be that the *hsp70* promoter is not activated rapidly enough or is not strong enough to make sufficient VLF-1 in transformed cells. It is suggested that a threshold level of VLF-1 is necessary to activate the *polh* promoter (Yang and Miller, 1998b). From this result, it is likely that a higher level of VLF-1 in this transformed cell line is required to support activation of *polh* promoter. The previous reports revealed that polyhedrin expression is tightly coupled with *vlf-1* expression and the level of VLF-1 is important for expression from the *polh* promoter (McLachlin and Miller, 1994; Yang and Miller, 1998a, b). In another reason, such a low effect of VLF-1 may be attributed to interactions between BmNPV -K1 VLF-1 and AcNPV *polh* promoter. Actually, The VLF-1 of BmNPV-K1 was different from amino acid sequences at 7 positions compared with AcNPV (Park *et al.*, 2000). The effect from this result is apparently unexplained but may reflect the reasons explained above.

We have shown that transformed insect cells, stably transformed with neomycin-resistance and *vlf-1* genes, can be selected easily and express foreign gene products of recombinant baculovirus rapidly at higher levels than the control Sf9 cells. Our results suggest that transformed cell line expressing VLF-1 may be utilized effectively for the foreign gene expression of recombinant baculovirus. Therefore, this transformed cell line could provide the model for the development of efficient insect cells.

Acknowledgments

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