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Production of Polyomavirus and Herpesvirus Recombinant Glycoproteins with Immunoreactivity Using a Rapid and Novel Expression System in Insect Cells for Applications in Vaccines and Serological Assays

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KEYWORDS: Polyomavirus; Herpes simplex virus, Varicella Zoster virus, Baculovirus expression system; Plasmid based Expression system; Recombinant protein.

ABSTRACT

Introduction: Although, conventional methods for the expression of polyomaviruses and herpesviruses recombinant proteins for serological assays and vaccine developments in baculoviruses are well established, the manipulations are laborious and time consuming. **Methods:** A new expression system based on plasmid was used to express two polyomaviruses major capsid protein VP1 (JCV VP1 and BKV VP1), and two herpesviruses glycoproteins (HSV-1 gD and VZV gE) in insect cells. A ligation independent cloning (LIC) was applied to generate the recombinant plasmids. Transfection of Sf9 insect cells were performed using the recombinants. The produced proteins were analysed using SDS-PAGE, immunofluorescence, and immunoblotting. **Results:** JCV-VP1, BKV-VP1, VZV-gE and HSV-1gD were successfully expressed in the insect cells, 48 h post-infection and detected in cytoplasm and cell membranes with immunoreactivity. This plasmid based expression system took 5 days to express the protein. **Conclusion:** The plasmid based expression system in insect cells was highly efficient and would be ideal for rapid expression of polyomaviruses and herpesviruses proteins in insect cells to be potentially used in applications such as vaccine components and serological assays.

Citation:

INTRODUCTION

Polyomaviruses and herpesviruses are among the most common of human viruses. They may cause some acute diseases including progressive multifocal leukoencephalopathy (PML) (JC polyomavirus) nephropathy, ureteric stenosis and haemorrhagic cystitis (BK polyomavirus), gastrointestinal tract, skin, ocular, genital and nervous system disorders (herpesviruses) [1,2]. There are some methods of vaccine development, including live attenuated vaccines, recombinant DNA and subunit vaccines which have been already applied for viral vaccines. However, producing recombinant proteins of herpesviruses and polymaviruses present difficult challenges for vaccine development against these pathogens [3].

Expression and serological assays have an important role in the diagnosis of viral infections, determining immunity to infections and vaccines development studies. The preparation of viral proteins through *in vitro* cell culture systems is well documented. However, for viruses with poor yield in culture such approaches may be inappropriate. Recombinant molecular techniques provide a better approach for the serological and vaccine development assays [4].The baculovirus expression system is one of the most popular techniques for the recombinant protein production. Many of the post translational modifications such as phosphorylation, glycosylation, acylation and proteolytic processing observed in mammalian cells are also observed in insect cells [5].

Despite the recent advances in baculovirus technology in view of cloning techniques and applied vectors and cell culture technology [4], the conventional baculovirus expression systems remain a time consuming and laborious methodology. This study provides a description of the use of a new expression method, namely, a plasmid based expression system called InsectDirect (Novagen, USA), for the rapid production of the major capsid protein VP1 of two polyomaviruses (*i.e.* BK & JC) [6], and glycoproteins of two herpesviruses (*i.e.* HSV-1gD [7] and VZV gE [8]).

MATERIALS AND METHODS

Generation of DNA Fragment

Entire gene sequences of JCV VP1 (NCBI accession number: J02226), BKV VP1 (NCBI accession number V01108), HSV-1 Gd (NCBI accession number JN555585) and

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VZV gE (NCBI accession number MH709377.1) from multiple isolates previously published in GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide&itool=toolbar) were aligned for each virus using BioEdit Sequence alignment Editor (Ibis Biosciences, Carlsbad, California, USA) and highly conserved regions were identified for each virus from which primers sequences were selected. A NCBI BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST/) search was utilized to indicate any homology between the primer sequences and all published sequences in Genbank. To generate specific vector-compatible overhangs, the sense primer incorporated a 5' CAGGGACCCGGT sequence and the anti-sense primer a 5' GGCACCAGAGCGTT sequence.

Using pJCV (1-4) plasmid containing the JC virus genome (American Type Culture Collection, ATCC: 45027) or BKV DNA extracted from a clinical sample as target, the JCV and BKV DNA regions encoding for the capsid protein VP1, was amplified by PCR. The sense primers were designated JCVVP1F (CAGGGACCCGGTATGGCCCCAACAAAAAGAAAAG) and BKVVP1F (CAGGGACCCGGTATGGCCCCAACCAAAAGAAAAG) and the antisense primers JCVVP1R (GGCACCAGAGCGTTTTACAGCATTTTTGTCTGCAACTG) and BKVVP1R (GGCACCAGAGCGTTTTAAAGCATTTTGGTTTGCAATTG). HSV-1 gD and VZV gE genes were generated by PCR from clinical samples using sense primers HSVgDF (CAGGGACCCGGTATGGGGGGGGCT) and VZVgEF (CAGGGACCCGGTATGGGGACAGTTAATAAA) and antisense primers HSVgDR (GGCACCAGAGCGTTCTAGTAAAACAAGGGCTGG) and VZVgER (GGCACCAGAGCGTTTCACCGGGTCTTATCTAT). The PCR products were purified using Microcon purification (Clear Diagnostic, Alamed, USA) and analysed by 2% agarose gel electrophoresis.

Recombinant Plasmid Construction

A ligation-independent cloning technique was applied as depicted in Fig. 1.

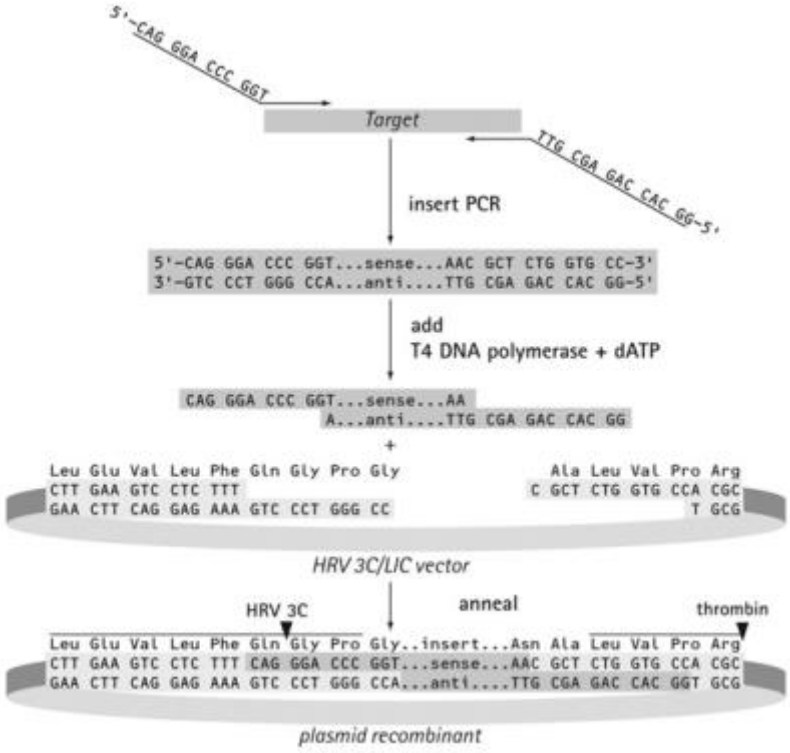

Fig. 1. 3C/LIC strategy diagram. After amplification with primers that included the indicated 5' LIC extensions, the PCR insert was treated with LIC-qualified T4 DNA Polymerase (+dATP) and was annealed to 3C/LIC vector.

Briefly, the insert with overhangs was prepared by PCR and then was treated with T4 DNA polymerase (Novagen, Merck, USA) and dATP (Novagen, Merck, USA). To create recombinants, the treated insert was annealed with the pIEx/Bac-3 3C/LIC vector (Novagen, Merck, USA). The annealed complex was used to transform *Escherichia coli* (NovaBlue GigaSingles™ Competent, K-12 strain; Novagen, Merck, USA). To confirm insertion of plasmid DNA colonies were screened by PCR after extraction of DNA. Vector-specific primers (IE1 Promoter and IE1 Terminator; Novagen, Merck, USA) were used, alternatively, one vector-specific primer was used in combination with a virus gene specific primer. For purification of recombinant plasmid, Mobius™ 200 kit (Novagen, Merck, USA) was used.

Transient Transfection of Insect Cells

Sf9 cells (Novagen, Merck, USA), derived from Spodoptera frugiperda, were grown with Bacvector medium (Novagen, Merck, USA). After transfecting 1 x10^6/ml Sf9 cells with 20 µg recombinant plasmid DNA, and 100 µl of Insect GeneJuice transfection reagent according to the InsectDirect expression protocol (Novagen, Merck, USA). The cells were incubated at 28°C with shaking (150 rpm) for 48 h.

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Cell Extraction and Protein Purification

For 1 ml of the culture volume, 50 µl of Insect PopCulture® reagent (Novagen, USA) was added to the cells, followed by 0.5 µl benzonase nuclease (Novagen, USA). The mixture was incubated for 15 min at room temperature. The culture extract was centrifuged for 10 min at 15,000 x g. The supernatant was saved. The expressed proteins were purified as previously described [9].

SDS-PAGE and Western Blot Analysis

Samples were applied to a NuPAGE Novex bis-Tris mini-gradient PAGE gel 4-12% (Invitrogen) and were run in SDS-Tris-glycerine buffer. The proteins were visualised by staining with Coomassie Blue (Simply Blue, Invitrogen). After SDS-PAGE, the proteins were electrophoretically transferred to PVDF (PVDF Filter paper sandwich, Invitrogen) using x cell (Invitrogen) at a constant voltage of 200 V. The PVDF membranes were then immersed in a solution of 5% dry skimmed milk-PBS-Tween 0.1% for 1 h. The membranes were then incubated for 1 h at room temperature in a 1:1500 dilution of mouse monoclonal to JCV VP1 (Abcam, Cambridge, UK); rabbit polyclonal to SV40 VP1 (Abcam); HSV gD-specific monoclonal; or mouse monoclonal to VZV gE (Abcam). After subsequent washing, the membranes were probed with a 1:1500 dilution of goat monoclonal to mouse IgG antibody conjugated to horseradish proxidase (Sigma) for JCV, HSV and VZV and with a 1:1500 goat polyclonal to rabbit IgG antibody conjugated to alkaline phosphatase (Abcam) for BKV. The blots were stained with 3, 3′-diaminobenzidine peroxidase substrate (Sigma) for JCV, HSV, and VZV and BCIP®/NBT Alkaline Phosphatase Substrate (Sigma) for BKV.

Dot Immunoblot

After spotting 30 µl of each sample to a 0.45µm-pore nitrocellulose membrane (Schleicher & Schuell, Germany), they were dried under vacuum using a dot blot apparatus (Vacuum Filtration System, Minifold, SRC, 96 D, Schleicher & Schuell, Germany). The air-dried membranes were then immersed in 5% skimmed milk and incubated with the first and second antibodies as described for the Western blots.

Immunofluorescence

Following application of 25 µl of the transfected insect cells of each spot of a PTFE coated 15-spot slide (C.A. Hendley, Essex), the slide was dried and immersed in cold acetone (-20°C) for 5 min. After washing in sterile water, a 1:100 dilution of anti-mouse JCV VP1, HSV-1 gD, or VZV gE antibodies was applied to each spot and incubated at 37°C for 30 min. After washing, the slide was incubated with a 1:100 dilution of anti-mouse FITC- conjugated (Dako, Denmark) at 37°C for 30 min and then examined by fluorescence microscopy.

RESULTS

Protein Analyses: (a) Immunofluorescence

To examine Sf9 cells transfected with the recombinant plasmids, an indirect immunofluorescence assay was performed (Fig. 2). At 48 h post infection (p.i.) VP1, gD and gE proteins could be observed in the cytoplasm and membrane of infected Sf9 cells. At 72 h p.i., optimum immunofluorescence was observed (Fig. 2A). Based on these observations, the time of 72 h p.i. was selected for the expression experiments.

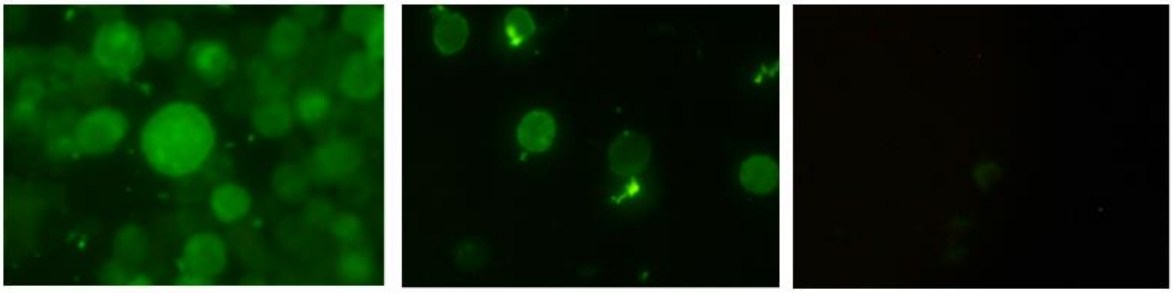

Fig.2. Immunofluorescence assay: **(A)** cells transfected with recombinant plasmid containing HSV-1 gD, 72 h p.i.; **(B)** cells transfected with recombinant plasmid containing BKV VP1, 72 h p.i. Panel C: cells not transfected with recombinant plasmid (negative control).

Protein Analyses: (b) SDS-PAGE and Western Blotting

Analysis of the expressed proteins by SDS-PAGE showed that the recombinant proteins of the expected size were produced by InsectDirect system. The peak of expression was obtained at day 5 p.i. in culture medium (Fig. 3). The synthesis of JCV-VP1, HSV-1gD and VZV-gE derivatives was confirmed by Western blot analysis using the viruses-specific antibodies. Western blotting analysis of the lysates of Sf9 cells infected with the recombinants revealed a band with an apparent Mw of 40 KDa representing JCV- VP1 (Fig. 4A) and BKV- VP1 (not shown) and 52 KDa for HSV-1 gD (not shown) and 95 KDa for VZV-gE (Fig.4B).

Dot Blot

The antigenicity of the recombinant proteins was further analysed by dot immunoblotting. All of the expressed proteins were found to react with the viruses specific antibodies (Fig. 5). The first row-extreme right dot in Panel A is more pronounced than the others because it represents Elute 1 and others are representing Elute 2.

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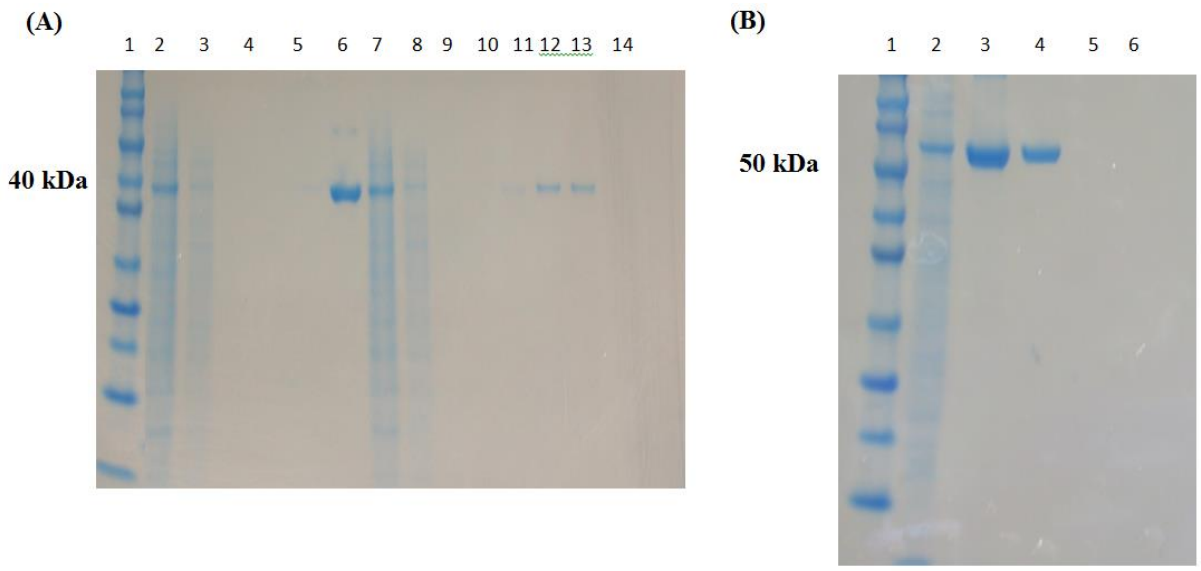

Fig. 3. Protein expression and purification analysed by SDS-PAGE. **(A) JCV (left)** and **BKV (right) VP1**: Lane 1: Mw Marker (EasyLadder, Bioline), Lanes 2 and7: cell supernatants; Lanes 3 and 8: flow-through; Lanes 4,5,9,10,11: wash; Lanes 6,12,13: elutes. **(B) HSV-1 gD:** Lane 1: Mw Marker (EasyLadder, Bioline); Lane 2: cell supernatant; Lanes 3 and 4: elute; Lanes 5 and 6: wash.

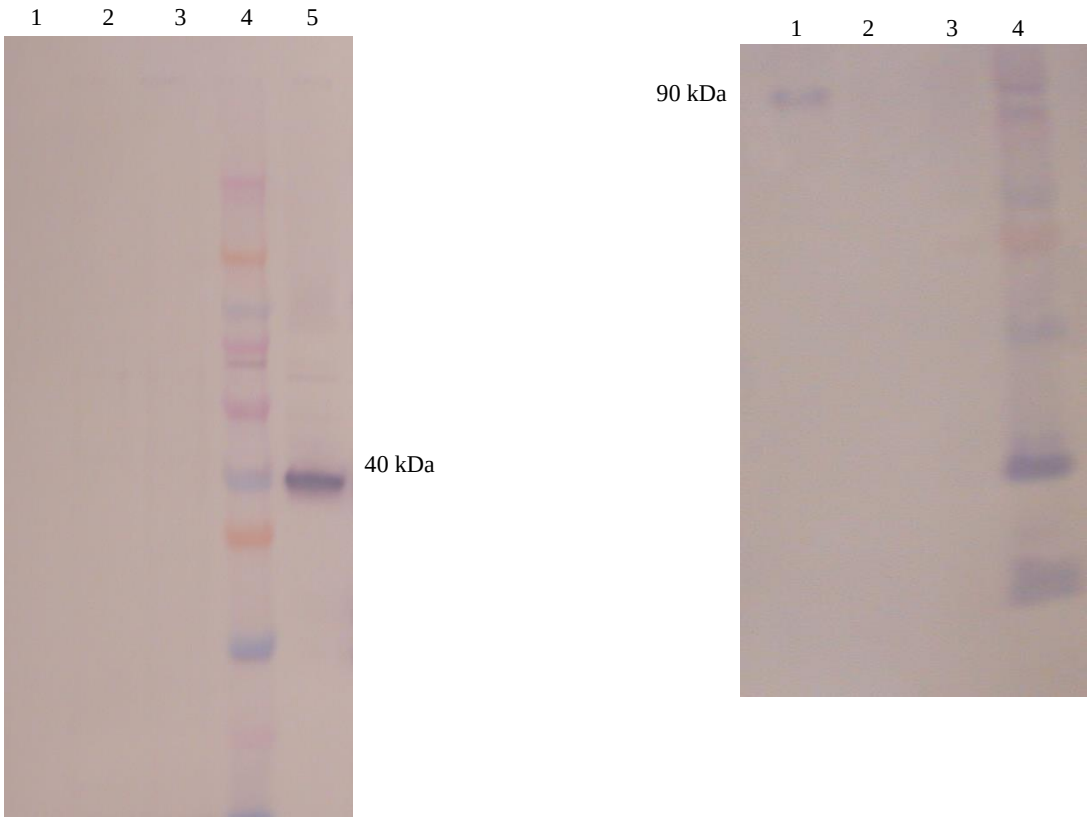

Fig. 4. Western blot analysis of the recombinant proteins. **(A) JCV-VP1**: Lane 1: Negative control; Lanes: 2 and 3 Wash; Lane 4: Mw Marker (EasyLadder, Bioline); Lane 5: recombinant infected cell lysate. **(B) VZV-gE:** Lane 1: recombinant infected cell lysate; Lanes: 2 and 3: Wash; Lane 4: Mw Marker (EasyLadder, Bioline).

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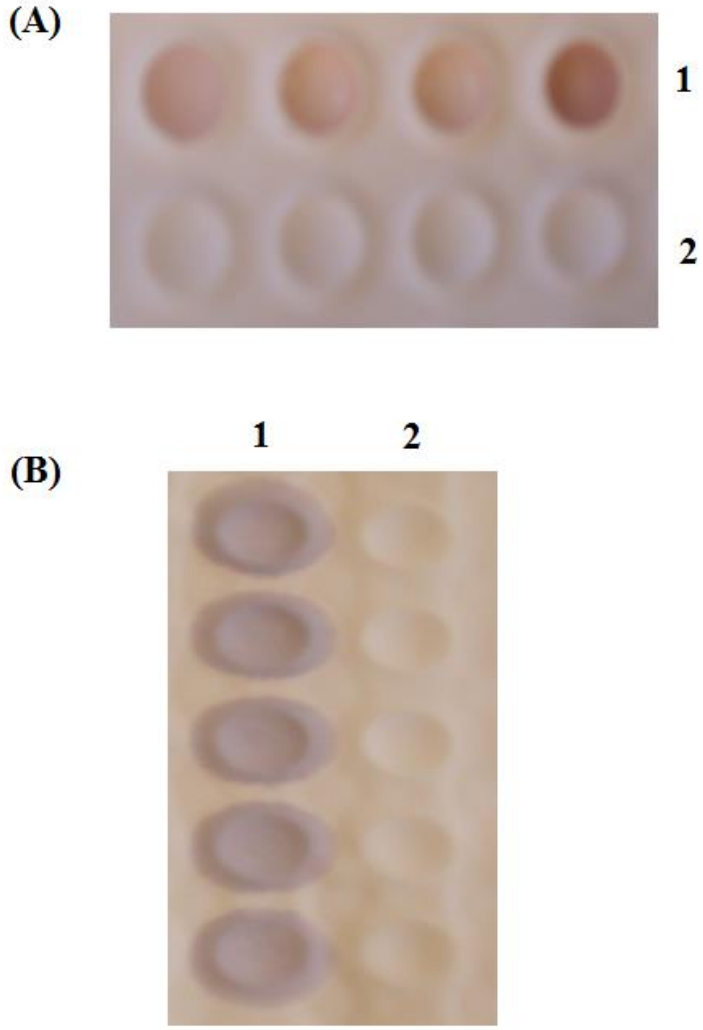

Fig.5. The immunoreactivity of the expressed proteins was examined using dot blot. **(A):** row 1: BKV VP1 with SV40 VP1 polyclonal antibody **(B):** column 1: HSV-1 gD with HSV gD specific monoclonal antibodies. The specificity of the reaction was confirmed by the lack of reactivity of these sera with the negative controls (non-infected cell lysates; row 2 in panel A & column 2 in panel B).

DISCUSSION

The expression of recombinant JCV and BKV VP1 and recombinant glycoprotein for HSV type 1 and VZV for use in vaccine development studies and serological assays has been demonstrated using both prokaryotic [10,11,12,13,14] and eukaryotic cells [15,16,1,3,2,17]. Prokaryotic expression offers some advantages in terms of protein yield, cost and ease of protein expression [12, 13]. However, the baculovirus expression system in insect cells takes advantage of post-translational modifications similar to those of eukaryotic cells which may be important in the expression of fully immunoreactive glycolysated antigens.

Previous studies aiming to express polyomavirus and herpesvirus recombinant protein in insect cells for use in vaccine development studies and serological assays have applied traditional baculovirus expression systems (10,11). The study presented here describe expression of polyomavirus and herpesvirus proteins in insect cells using a plasmid expression system. The cloning method utilized here provides a directional cloning strategy to quickly create recombinants. The virus gene and promoter are carried by PIEX/Bac vector. The vector features the hr5 (homologous region 5) enhancer, the ie1 (immediate early 1) promoter and the AcNPV p10 very late promoter from Autographa californica nuclear polyhedrosis virus (AcNPV) for both plasmid and baculovirus-mediated expression (9). Therefore, it is easy to switch between the different expression systems to meet requirements for protein production. In the plasmid-mediated system, the promoter/enhancer combination uses endogenous insect cell transcriptional machinery. The vector is designed to express the target protein immediately downstream of an HRV 3C protease cleavage site. The immunofluorescence analysis of infected cell showed that polyomavirus VPI, and herpesvirus gD and gE proteins were expressed successfully in insect cells. The synthesis of recombinants was further analyzed using SDS-PAGE and immunoblotting.

We found that JCV VP1, BKV VP1, HSV-1 gD and VZV gE could be produced in insect cells from a plasmid based expression as observed previously in baculovirus systems [15,16,3,2,17]. A time course study of polyomavirus VP1 protein expression in this system revealed that the peak of VP1 protein expression in culture was 5 days p.i which is in line with the observation of protein expression using conventional recombinant baculovirus based expression systems. In conclusion, the current study demonstrates the possibility of successful expression of recombinantVP1, HSV-1 gD and

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VZV-gE recombinant proteins for downstream applications such as being used as vaccine components or serological studies. Moreover, it confirms that the PIE/Bac 3LIC vector holds promise as a tool for rapid directional cloning. The system is more efficient than the baculovirus expression system by elimination of the time consuming steps of recombinant baculovirus construction, screening, amplification, and plaque purification.

ACKNOWLEDGEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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