
Baculovirus Expression Vectors that Incorporate the Foreign Protein into Viral Occlusion Bodies

BioTechniques 34:81-87 (January 2003)

ABSTRACT

Current baculovirus expression systems typically produce soluble proteins that accumulate within the infected insect cell or are secreted into the growth medium. A system has now been developed for the incorporation of foreign proteins, along with the matrix protein, polyhedrin, into baculovirus occlusion bodies. Initial studies showed that a recombinant virus expressing a translational fusion between polyhedrin and GFP did not form occlusion bodies. However, a baculovirus coexpressing native polyhedrin and

the polyhedrin-GFP fusion protein formed occlusion bodies that fluoresced under UV light, demonstrating that they included the polyhedrin-GFP fusion protein. This was confirmed by immunoblot analysis. Thus, incorporation of a foreign protein into occlusion bodies depends on an interaction between native polyhedrin and the polyhedrin fusion protein. Electron microscopy demonstrated that the occlusion bodies containing GFP also incorporated virions as expected. These ColorPol occlusion bodies were as infectious to insect larvae as occlusion bodies produced by wild-type virus. This new system expands the capabilities for foreign gene expression by baculoviruses, which has implications for biopesticide design, novel vaccine delivery systems, and fusion protein purification applications.

INTRODUCTION

Baculoviruses are a large family of

viruses that mostly infect lepidopteran insects. A characteristic feature of baculoviruses is the formation of large proteinaceous occlusion bodies, called polyhedra, in which the virions are embedded. Polyhedra protect the virus in the environment and are required for horizontal spread between hosts. They can be up to 15 μm in diameter. The matrix of these bodies is composed largely of the protein polyhedrin. This is highly abundant, comprising up to 50% of the protein in an infected cell (8,13,14). Several studies have examined factors affecting polyhedra morphogenesis (2-5,7,17). However, many aspects of occlusion body assembly remain unclear.

Baculoviruses are of considerable interest because of their potential as biological insecticides and their use as eukaryotic gene expression vectors (12). Their development as an expression vector system was prompted by the fact

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that the polyhedrin gene is both highly expressed and nonessential for viral replication in cell culture. Thus, early expression vector systems typically involved the replacement of the polyhedrin gene with the gene of interest placed under the control of the polyhedrin promoter. This gave rise to recombinant viruses that overproduced the foreign protein in cell culture but that could not produce occlusion bodies. More recently, expression vector systems have been developed that generate recombinant viruses that are able to produce occlusion bodies (13). Such viruses are particularly useful for producing the foreign protein in infected insects.

A wide variety of variants of these basic systems has been developed that allows the use of different promoters or provide more rapid screening systems for the identification of the recombinant viruses. Typically, the foreign protein is produced in a soluble form and is directed to its appropriate subcellular or extracellular location. Where the virus is capable of producing occlusion bodies, these are normal viral polyhedra and do not contain the foreign protein. However, there are circumstances where it could be advantageous to incorporate a foreign protein directly within the polyhedrin matrix to produce recombinant polyhedra. For example, it could be useful to engineer polyhedra to carry an insect toxin for improved biopesticidal activity or to display foreign proteins as a vaccine delivery tool for vertebrates (6). An early study by McLinden et al. (11) demonstrated that the polyhedrin protein could be manipulated to encode small epitopes of foreign proteins. However, the number and the location of these additional amino acids affected whether successful assembly of recombinant polyhedra occurred.

Here we describe the production of a recombinant baculovirus in which a foreign protein, GFP, is actually incorporated into viral polyhedra. Polyhedra that incorporate this full-length protein retain the ability to occlude virions. These occlusion bodies are infectious to insects and fluoresce under UV light, demonstrating that GFP remains functional. This technology expands the range of applications of the baculovirus expression system.

MATERIALS AND METHODS

Cell Lines and Viruses

Spodoptera frugiperda IPLB Sf21-AE (16) clone 9 (Sf9) cells were maintained at 27°C in TC100 medium supplemented with 10% heat-inactivated FBS. All viruses were propagated and titered in Sf9 cells (13,14). Polyhedra and viral DNA were obtained from Sf9 cells by standard methods (13,14).

Recombinant Transfer Vectors

The 730-bp GFP gene from pGFP (BD Biosciences Clontech, Palo Alto, CA, USA) was inserted into the *Bam*HI and *Pst*I sites of pBacPAK8 (BD Biosciences Clontech) to yield pAcG (Figure 1A). A 903-bp region from AcMNPV polyhedrin was amplified using the primers 5'-GCCATTGTAATGAGACG-CAC-3' and 5'-AACTCGAGATACGC-CGGACCAGTGAAC-3'. The latter replaces the termination codon TAA with an *Xho*I site (underlined). All PCRs comprised 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The product was digested with *Eco*RV and *Xho*I and inserted into pBacPAK8 to yield pBacPAK-AcPOL. GFP was amplified from pGFP using the primers

5'-AACTCGAGATGAGTAAAGGAG-AAGAAC-3' and 5'-CTATTTGTAT-AGTTCATCCATGCC-3'. The amplified product was blunt-ended with Klenow, digested with *Xho*I site (underlined), and inserted into *Xho*I- and *Sma*I-cut pBacPAK-AcPOL to yield pAcPG (Figure 1B). To construct pColorPol, a 1605-bp fragment of the polyhedrin-GFP fusion from pAcPG was amplified with 5'-AAAAAAAAGATCTACCATGCCGGATTATTC-3' and 5'-CGCTC-TAACATAACCACCC-3' corresponding to the translational start and 3' untranslated regions of polyhedrin. The amplified fragment was digested with *Bg*III (underlined) and *Sna*BI and inserted into *Bam*HI- and *Sna*BI-cut pAcUW31 (BD Biosciences Clontech), giving pAcUW-PG. AcMNPV polyhedrin was amplified with 5'-AAAAAAAAGATCTACCATGCCGGATTATTC-3' and 5'-GT-AGAATTCATTAATACGCCGACC-AG-3'. These include *Bg*III and *Eco*RI sites (underlined). The amplified gene was cloned into pAcUW-PG to give pColorPol (Figure 1C).

Construction of Recombinant Viruses

One microgram of BacPAK6 viral DNA (BD Biosciences Clontech) and 5

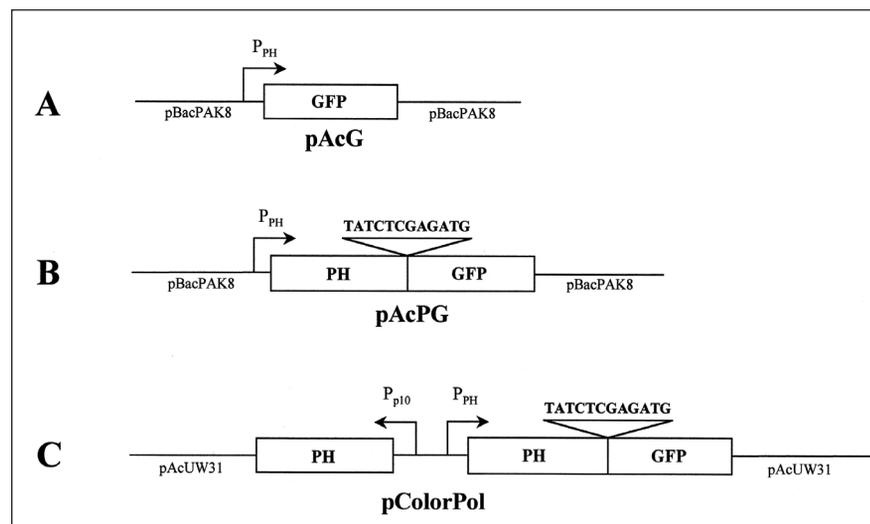


Figure 1. Transfer vectors for the production of recombinant baculoviruses. (A) The transfer vector pAcG was constructed by insertion of the GFP gene (GFP) into pBacPAK8, under the control of the polyhedrin promoter (P_{PH}). (B) The transfer vector pAcPG was constructed by insertion of the GFP gene as a translational fusion at the 3' end of the AcMNPV polyhedrin gene (PH), under the control of the polyhedrin promoter (P_{PH}). The transfer vector pAcUW31 was used for the construction of the transfer vector pColorPol (C). The AcMNPV polyhedrin gene and the fusion gene polyhedrin-GFP were introduced under the control of p_{10} (P_{p10}) or polyhedrin (P_{PH}) promoters, respectively. The arrows show the direction of transcription.

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μ g transfer vector DNA were cotransfected into Sf9 cells by lipofection as described (13). The virus-containing supernatant was harvested five days later, and recombinant AcMNPV was isolated by plaque purification (13,14). The genome structures of the recombinant viruses were confirmed by PCR, restriction endonuclease analysis, and Southern blotting (data not shown).

SDS-PAGE and Immunoblot Analysis

Sf9 cells were mock-infected or infected with wild-type or recombinant AcMNPV at an MOI of 5 pfu/cell, and harvested three days post-infection. Total cellular lysates were subjected to 10% SDS-PAGE (9), electroblotted, and incubated with anti-GFP (BD Bio-

sciences Clontech) or anti-AcMNPV polyhedrin antibody. The anti-AcMNPV polyhedrin antibody was generated in mice by injection of purified AcMNPV polyhedrin protein. Bound antibodies were visualized by incubation with goat anti-mouse IgG conjugated with alkaline phosphatase [1:1000 (v/v); Sigma, St. Louis, MO, USA) as described previously (15).

Immunogold Staining

Samples were fixed for 3 h in 4% formaldehyde, 1% glutaraldehyde in 0.1 M sodium phosphate (pH 7.5) containing 0.15 mM CaCl_2 and 0.45 M sucrose (FM), and incubated overnight in FM, pH 10.4, without glutaraldehyde. The samples were rinsed in 0.1 M sodium phosphate (pH 7.5), dehydrated in a graded ethanol series (up to 95%), and embedded in Lowicryl K4M (Polysciences, Warrington, PA, USA). Ultra-thin sections mounted on formvar-coated nickel grids were treated for 10 min with TBS (0.02 M Tris-HCl, pH 7.5, containing 0.5 M NaCl). The sections were etched with 3% H_2O_2 in double-distilled water for 5 min and blocked with 3% BSA in TBS for 30 min. Double immunogold staining was performed as described previously (1). The sections were incubated with primary antiserum against GFP (diluted 1:200), washed in TBST three times for 15 min with gentle agitation, and exposed to gold-conjugated goat anti-mouse IgG (30 nm; BioCell, Cardiff, UK) diluted 1:10 in TBST for 60 min. After washing with 0.3% BSA in TBS, the grids were dried, and the reverse of the grid was incubated with antiserum against polyhedrin (1:300) and gold-conjugated goat anti-mouse IgG (10 nm; Zymed, CA, USA) diluted 1:30 in TBST.

Bioassays

The infectivity and virulence of ColorPol were compared to wild-type AcMNPV in the Diamondback moth, *Plutella xylostella*. *P. xylostella* larvae were obtained from a laboratory colony and reared at 25°C on Chinese cabbage under a 16 h:8 h light dark cycle. To determine the median lethal dose (LD_{50}), 128 third-instar larvae were fed six dos-

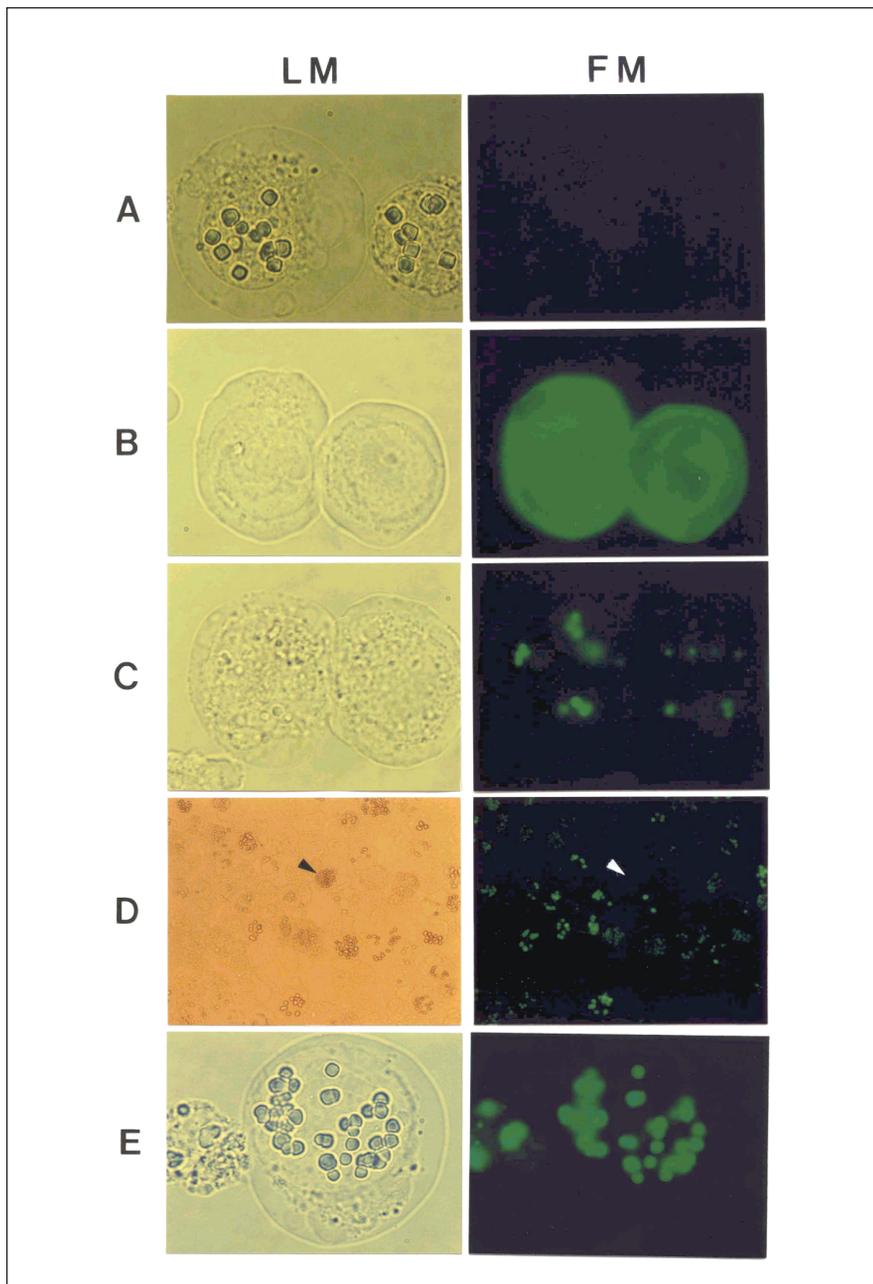


Figure 2. Polyhedra formation by AcMNPV recombinants. Sf9 cells infected with wild-type AcMNPV (A, $\times 1000$), AcG (B, $\times 1000$), AcPG (C, $\times 1000$), both wild-type AcMNPV and AcPG (D, $\times 200$), or ColorPol (E, $\times 1000$) were observed four days post-infection by light (LM) and fluorescent (FM) microscopy. Arrows in panel D indicate non-fluorescent polyhedra.

Table 1. LD₅₀ and ST₅₀ Values of ColorPol and Wild-Type AcMNPV against *P. xylostella* Larvae

Virus	LD ₅₀			ST ₅₀		
	n*	Polyhedra/ Larva	95% CI	n*	Hours	95% CI
AcMNPV	768	2798	1483–4884	128	92.8	88.0–98.0
ColorPol	768	4907	3143–8366	128	108.7	105.5–112.3

*Number of insects

es (20 480, 10 240, 5120, 1280, and 640 polyhedra/larva) of AcMNPV or ColorPol on a small plug of Chinese cabbage leaves. Larvae that consumed the dose in 24 h were transferred to fresh Chinese cabbage leaves and examined twice daily. Cadavers were removed, and the cause of death was confirmed. The data were analyzed by probit analysis. To determine the median survival time (ST₅₀), 128 third-instar larvae were fed 10 240 polyhedra/larva of wild-type AcMNPV or ColorPol (approximately equivalent to an LD₉₀ dose) on a small plug of Chinese cabbage leaves. After feeding, the larvae were transferred to fresh diet and observed as before. ST calculations were made using the vistat program (Boyce Thompson Institute, Ithaca, NY, USA). The 95% confidence intervals (CI) were also calculated for the LD₅₀ and ST₅₀ values.

RESULTS

Formation of Polyhedra Incorporating GFP

The structures of the transfer plasmids used to generate the recombinant viruses employed in this study are summarized in Figure 1. The virus AcG was generated by cotransfection of parental *Autographa californica* nucleopolyhedrovirus (AcMNPV) with the transfer plasmid pAcG (Figure 1A) and therefore expresses GFP in place of polyhedrin under the control of the polyhedrin promoter. This represents the classical approach to the generation of a baculovirus expression vector. As expected, AcG did not form polyhedra, but GFP was detected in a soluble form in the nucleus and cytoplasm of infected cells (Figure 2B). The first approach we attempted to generate a virus that

produces occlusion bodies incorporating GFP was to express a polyhedrin-GFP fusion protein. The transfer plasmid pAcPG includes the GFP gene fused in frame to the 3' end of the polyhedrin gene (Figure 1B). The fusion protein is under the control of the polyhedrin promoter. However, the recombinant virus generated, AcPG, still did not form polyhedra, unlike wild-type viral infection (Figure 2A). GFP was now detected exclusively in the nucleus, apparently in a particulate form, but these particles were distinct from polyhedra (Figure 2C).

We reasoned that the formation of normal occlusion bodies might require the presence of unfused polyhedrin protein. Thus, we tried coinfecting cells with both wild-type AcMNPV and AcPG. In this case, occlusion body formation was observed (Figure 2D). In most cells, the polyhedra were fluorescent under UV light, indicating incorporation of GFP. A small proportion of cells contained non-fluorescent polyhedra. Cells coinfecting with wild-type AcMNPV and AcG produced non-fluorescent polyhedra (data not shown). We suspected that in the AcMNPV-AcPG coinfection (Figure 2D) a few cells had been infected with wild-type AcMNPV only, producing non-fluorescent polyhedra, whereas most cells had been infected with both viruses and produced

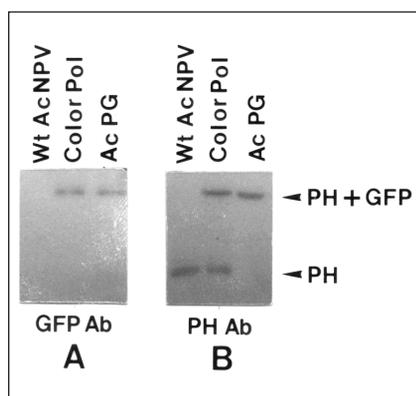


Figure 3. Immunoblot analysis of Sf9 cells infected with ColorPol. At three days post-infection, total cellular lysates were separated by 10% SDS-PAGE and immunoblotted with anti-GFP (A) or anti-polyhedrin (B) antibody. Wild-type AcMNPV and AcPG served as controls. The positions of polyhedrin (PH) and the polyhedrin-GFP fusion protein (PH + GFP) are indicated.

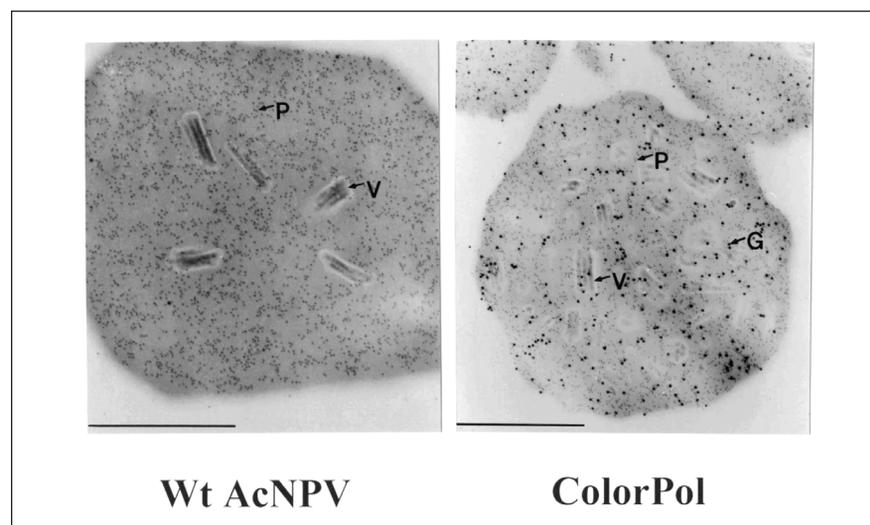


Figure 4. Immunogold labeling of recombinant polyhedra. Purified polyhedra formed by wild-type AcMNPV or ColorPol (left and right panels, respectively) were analyzed by immunogold labeling with a mixture of antisera against polyhedrin and GFP. Gold particles attached to the anti-polyhedrin (P, 10 nm) and anti-GFP (G, 30 nm) antibodies were observed by transmission electron microscopy. Virions (V) are indicated. The bars represent 1 μm.

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fluorescent polyhedra. This suggested that some native polyhedrin protein is required for occlusion body formation. Therefore, we constructed the virus ColorPol which coexpresses native polyhedrin and the polyhedrin-GFP fusion (Figure 1C). In this virus, the polyhedrin-GFP fusion protein is expressed under the control of the polyhedrin promoter, whereas the p10 promoter drives the polyhedrin expression. P10 is another abundantly produced baculovirus protein that is expressed with very similar kinetics to the polyhedrin gene (13). All ColorPol-infected cells produced fluorescent polyhedra (Figure 2E).

Characterization of Polyhedra Displaying GFP

Protein synthesis in wild-type AcMNPV-, ColorPol-, and AcPG-infected Sf9 cells was analyzed by immunoblotting (Figure 3). Both polyhedrin and

GFP antisera detected the polyhedrin-GFP fusion as a 60-kDa protein in ColorPol- and AcPG-infected cell lysates. The polyhedrin antibody detected polyhedrin in ColorPol- and wild-type AcMNPV-infected cells. Purified ColorPol polyhedra also comprised the fusion protein and native polyhedrin (data not shown). To verify the presence of GFP in ColorPol polyhedra, occlusion bodies were examined by immunogold labeling (Figure 4). Labeling due to polyhedrin and GFP was homogeneously distributed throughout the occlusion bodies. These data also showed that the occlusion bodies are morphologically normal and contained virions, suggesting that they might be infectious. This was confirmed by bioassay of ColorPol occlusion bodies fed to *P. xylostella* larvae. The infectivity of the ColorPol virus was not significantly different from that of wild-type AcMNPV, but insects infected by ColorPol

displayed a 17% increase in survival time (Table 1). PCR analysis of ColorPol virus recovered from infected insects confirmed the recombinant locus is stable (data not shown).

DISCUSSION

Our initial attempt to produce occlusion bodies displaying GFP entailed the generation of a recombinant virus expressing a polyhedrin-GFP fusion protein. The fusion protein was detected exclusively in the nucleus, whereas GFP alone was present throughout the cell. Thus, sequences in polyhedrin are responsible for the retention of the fusion protein in the nucleus. Nuclear localization signals in polyhedrin have been described previously (7). The polyhedrin-GFP fusion protein did not form occlusion bodies but was detected in a particulate form, suggesting that

some assembly had occurred. McLinden et al. (11) reported that polyhedrin proteins with short insertions in the central part of the protein either failed to form occlusion bodies or gave rise to abnormal occlusion bodies. Insertion of the foreign amino acids at the N-terminus gave rise to morphologically normal occlusion bodies that did not occlude virus efficiently.

We chose a different strategy of co-expressing native polyhedrin with the fusion protein. This virus, ColorPol, coexpresses native polyhedrin and a polyhedrin fusion protein. ColorPol occlusion bodies are as infectious to insects as their wild-type counterparts, indicating that the incorporation of GFP does not affect dissolution of the occlusion bodies in the gut of the insect. Surprisingly, the speed of kill of the virus seemed to be somewhat slower than that of wild-type virus (Table 1). It is not clear whether this is due to alterations in the viral polyhedra or to the presence of GFP in the infected cells.

A major application of recombinant baculovirus technology has been to produce viruses with improved insecticidal properties. The technology we have described facilitates the development of recombinant baculoviruses that can deliver insecticidal proteins that are active in the gut of the insect. We have recently exemplified this approach by the generation of a recombinant virus that produces occlusion bodies containing an insecticidal *Bacillus thuringiensis* crystal protein toxin (ColorBtrus). This was based on the construction of a polyhedrin-Bt toxin-GFP fusion polypeptide (8). Both the infectivity and speed of action of ColorBtrus occlusion bodies are dramatically enhanced compared to wild-type virus (Je et al., submitted).

This technology should also facilitate other novel applications of baculovirus expression vectors. For example, it should represent an advantageous approach to the expression of proteins to be used as subunit vaccines. The delivery of immunogens in a particulate form should result in the development of a robust inflammatory (T_{H1}-mediated) response in vertebrates. The presence of other viral proteins in the occlusion body will also facilitate this. The ability to incorporate simultane-

ously more than one protein into the polyhedra, as we have done for ColorBtrus, raises the possibility of using this approach for the delivery of multivalent subunit vaccines or for the delivery of immune regulatory proteins along with the immunogen. Another possible application of this technology is the production of insoluble bait protein for the capture of interacting proteins by panning. We are currently exploring these and other applications of the technology.

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This research was supported in part by the Brain Korea21 Project. S.K.K. is deceased. Address correspondence to Dr. Yeon Ho Je, School of Agricultural Biotechnology Seoul National University, Suwon 441-744, Korea. e-mail: btrus@hanmail.net

Received 18 June 2002; accepted 10 October 2002.

Y.H. Je^{1,4}, B.R. Jin², H.W. Park¹, J.Y. Roh¹, J.H. Chang¹, S.J. Seo³, J.A. Olszewski⁴, D.R. O'Reilly⁴, and S.K. Kang¹
¹Seoul National University Suwon, Korea
²Dong-A University Pusan, Korea
³Gyeongsang National University Jinju, Korea
⁴Imperial College of Science, Technology, and Medicine London, UK

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