Recombinant baculoviruses as expression vectors for insect and mammalian cells

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Baculovirus expression vectors are widely used for expressing heterologous proteins in cultured insect cells. Recent advances include further development of the system for production of multi-subunit protein complexes, co-expression of protein-modifying enzymes to improve heterologous protein production, and additional applications of baculovirus display technology. The application of modified baculovirus vectors for gene expression in mammalian cells continues to expand.

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Abbreviations

AAV	adeno-associated virus
CMV	cytomegalovirus
HBV	hepatitis B virus
HCV	hepatitis C virus
HSV-1	herpes simplex virus type 1
rBV	recombinant baculovirus
scFv	single chain antibody fragment
VLP	virus-like particle

Introduction

Baculovirus expression vectors continue to be used extensively for the expression of a variety of recombinant proteins in insect cells. These include cytosolic, nuclear, mitochrondrial, membrane bound and secreted proteins. Recombinant baculovirus (rBV) infected insect cells also provide a useful system for studying the viral particle assembly processes. This system is being increasingly utilized for the development of vaccine candidates based on the production of virus-like particles (VLPs) and conventional recombinant antigens. Co-expression of protein-modifying enzymes using multiple rBVs can be used to enhance the production of functional recombinant proteins produced by infected insect cells. Baculovirus surface display continues to evolve as a useful research tool. An emerging area is the application of modified baculoviruses, containing mammalian promoter elements, for the efficient transient and stable transduction of diverse mammalian cell types. This review covers recent advances in the application of rBVs in these areas.

Production of virus-like particles and vaccine antigens

Baculovirus expression vectors continue to prove valuable for the production and characterization of VLPs. Viruses encoding multiple heterologous viral proteins or a panel of viruses, each encoding a single heterologous protein, can be used to infect insect cells. In addition to allowing one to study viral assembly processes in the absence of infectious virus, the production of VLPs is proving valuable for the safe production of novel vaccines.

rBVs have proven to be powerful tools for investigating the capsid assembly process of herpes simplex virus type 1 (HSV-1). Intermediates in the HSV-1 capsid assembly process have been identified in studies using a cell-free assembly system [1]. A panel of rBVs encoding HSV-1 capsid proteins have been used to infect insect cells. Mixing of extracts from the rBV-infected cells and incubating the mixture *in vitro* results in capsid assembly. To determine the potential involvement of insect cell-encoded proteins in capsid formation, Newcomb *et al.* [2] purified HSV-1 capsid proteins from rBV-infected cells and tested the ability of the purified proteins to assemble into procapsids *in vitro*. These studies demonstrated that procapsids can be readily formed by the *in vitro* assembly of purified capsid proteins in the absence of additional insect cell factors. The major structural viral glycoprotein (VP1) of the human polyomavirus JC virus, the causative agent of progressive multifocal leukoencephalopathy, has been produced using rBVs [3]. The recombinant VP1 formed VLPs with the typical morphology of empty JC virus particles. The purified VLPs were highly immunogenic when administered with adjuvant. Hyperimmune serum from vaccinated rabbits inhibited binding of VLPs to host cells and was capable of neutralizing a natural JC virus isolate. Interestingly, a 4.5 kb pCMV-β-galactosidase plasmid DNA could be efficiently packaged into VP1 VLPs, and the packaged DNA was successfully transferred into COS-7 cells as demonstrated by the expression of β-galactosidase. Thus, the VP1 VLP may prove useful for both vaccine development and as a gene delivery system.

Ke *et al.* [4] have studied the assembly of polyomavirus capsid-like particles using combinations of rBV that encode the viral structural proteins VP1, VP2 and VP3. Similar to the results with JC virus, recombinant capsid-like particles could be purified from insect cells infected with an rBV producing VP1 alone. Expression of VP2 and VP3 alone did not result in the formation of particles; however, these proteins were incorporated into the particles formed in cells producing VP1. In all cases, cellular DNA fragments of about 5 kb in size were packaged into the VLPs. Expression of VP2, the major component of the virus capsid of parvoviruses, in rBV-infected insect cells resulted in the production of VLPs [5]. The parvovirus VLPs were highly immunogenic in the absence of adjuvant and conferred protective immunity to the vaccinated host. In addition, the potential use of the VLPs as an antigen delivery system was demonstrated by incorporating

either a $CD8^+$ epitope from lymphocytic choriomeningitis virus or a C3:T epitope from poliovirus into the VLP. Adler *et al.* [6] have used rBVs to produce bluetongue virus core-like particles expressing a T-cell epitope of the M1 protein of influenza A virus. The rBV system has also been used for the production of rotavirus VLPs [7]. Vaccination with the purified rotavirus VLPs resulted in protective immunity against a live heterotypic rotavirus challenge. To determine if the host range of a B-lymphotropic papovavirus virus could be altered, RGD (amino acid single letter code) motifs were inserted into sites predicted to be exposed on the viral surface of the VP1 protein and VLPs were produced using rBV [8]. RGD motifs are common in cell–cell and cell–matrix interactions, mediating the binding of a wide range of proteins. Insertion of the RGD sequence into a number of sites was tolerated; however, it was not determined if the host range of the modified VLP was altered.

A number of recent studies [9–11] have also demonstrated the utility of rBVs for producing retroviral- and lentiviral-like VLPs. This may represent a promising approach for the development of an HIV/AIDS vaccine candidate. An interesting approach to producing a multistage *Plasmodium falciparum* candidate vaccine has been described by Shi *et al.* [12•]. A synthetic gene encoding 21 epitopes derived from nine stage-specific *P. falciparum* antigens was expressed in rBV-infected insect cells. Immunization of rabbits with the purified antigen in the presence of different adjuvants generated antibody responses that recognized vaccine antigen, linear peptides contained in the vaccine, and all stages of *P. falciparum*. Studies in a mouse model system using rBV-produced influenza A virus hemagglutinin and neuraminidase have shown that production of these antigens in insect cells may offer an improved alternative to conventional influenza vaccines currently produced in embryonated chicken eggs [13].

Enhancement of functional protein production by rBV-infected insect cells

A variety of approaches continue to be explored for improving the yield of correctly processed, functional proteins using the rBV protein expression system. Hollister *et al.* [14•] have established an Sf9 insect cell line that constitutively expresses bovine α1,4-galactosyltransferase from the baculovirus immediate early promoter. These cells support normal levels of baculovirus replication. rBV infection stimulated α1,4-galactosyltransferase activity by almost fivefold 12 hours post infection, followed by a gradual decline in enzyme activity. Infection of these cells with an rBV encoding tissue plasminogen activator resulted in the production of a galactosylated end-product. Expression of a functional secreted form of the ectodomain of the lutropin receptor was enhanced when placed under control of the p10 promoter as compared to that of the polyhedrin promoter [15]. The ectodomain produced under the control of the p10 promoter displayed a more complex glycosylation profile. The weaker p10 promoter may not overwhelm the processing capacity of the endoplasmic reticulum as compared to the stronger polyhedrin promoter. Kulaskosky *et al.* [16] have examined the extent of *N*-glycosylation of secreted placental alkaline phosphatase produced by rBV-infected *Trichoplusia ni* and *Spodoptera frugiperda* cell lines. The *S. frugiperda* cells produced more fucosylated oligosaccharides than either of the *T. ni* cell lines studied.

A limitation of the baculovirus expression system is its inefficiency at properly processing heterologous proteins that are initially synthesized as larger inactive precursor proteins, such as peptide hormones, neuropeptides, growth factors, matrix metalloproteases and fusogenic viral envelope glycoproteins. The responsible processing enzymes have been designated proprotein convertases. A number of studies have shown that co-expression of the prohormone convertase furin enhances the production of the correctly processed protein. Recent studies have demonstrated that co-expression of furin with transforming growth factor β1 results in a 7.8-fold increase in the production of mature active growth factor [17]. The proprotein cleavage of the cellular adhesion protein E-cadherin has also been achieved using an rBV furin co-expression system [18]. The gene encoding an Sf9 cell enzyme homologous to furin has recently been cloned and characterized [19•]. The substrate specificity and inhibitor profiles are identical for the insect-cell enzyme and mammalian furin, whereas calcium-dependence, pH-optimum, and thermostability differ. Interestingly, the cleavage of recombinant influenza virus hemagglutinin was significantly enhanced by Sf9 cells after rBV-directed overexpression of Sf9 furin. Thus, in many cases the inability of insect cells to completely process overexpressed precursor proteins is probably due to an insufficient quantity of the insect cell furin-like enzyme.

The secretion of heterologous IgG proteins by rBV infected insect cells is accompanied by the accumulation of insoluble immunoglobulin in the infected cells. Co-expression of the cytosolic chaperone hsp70 increased the solubility of intracellular immunoglobulin [20] and led to an increase in the amount of secreted immunoglobulin. It will be interesting to determine the effect of co-expressing hsp70 and the chaperone BiP [21] on immunoglobulin secretion. A comparison of the action of cytosolic hsp70 to the endoplasmic reticulum BiP suggests a sequential action in which hsp70 increases the solubility of pre-processed immunoglobulin, whereas BiP enhances the solubility of processed immunoglobulin chains. An attempt to increase the amount of recombinant single-chain antibody fragment (scFv) secreted from rBV-infected cells was made by co-expressing a bacterial signal peptidase together with the antibody fragment [22]. The production of secreted scFv was enhanced 3.5-fold three days post infection in the presence of the signal peptidase; however, it is not clear whether the increase in secreted scFv was due to increased secretion or cell lysis

due to toxicity of the expressed peptidase. Golden *et al.* [23] investigated the effect of promoters and signal sequences on the production of HIV-1 gp120 in the rBV expression system. Production of secreted gp120 in Sf9 cells was comparable using either the polyhedrin promoter or a hybrid late–very late promoter. Fusion of the human tissue plasminogen activator signal sequence to gp120 yielded the highest level of secreted protein as compared to fusion to the human placental alkaline phosphatase or baculovirus envelope glycoprotein gp67 signal sequences. Expression of higher levels of functional μ-opioid receptor was achieved by including a 209 nucleotide sequence of the 5′ untranslated region of the receptor in the rBV vector as compared to a truncated 11 nucleotide sequence [24]. Interestingly, a TAAG sequence is located 118 bp upstream of the start codon of the μ-opioid receptor and thus may serve to enhance transcription of μ-opioid receptor mRNA. A feature of all characterized late and very late baculovirus transcripts is their initiation within the conserved sequence TAAG. Duffy *et al.* [25•] have shown that the rBV system can be used for the site specific, enzymatic biotinylation of recombinant proteins in insect cells using biotin acceptor peptides and co-expression of the *Escherichia coli* biotin holoenzyme synthetase (BirA). This system should prove very useful for site-specific biotin labeling of recombinant proteins produced using rBV vectors.

Baculovirus display technology

The expression of foreign proteins on the baculovirus surface has been demonstrated previously [26–28]. A recent study by Ernst *et al.* [29•] demonstrated the applicability of baculodisplay technology to the generation and screening of eukaryotic expression libraries. Variations of the HIV-1 gp41 epitope 'ELDKWA' were inserted into the antigenic site B of influenza virus hemagglutinin expressed on the surface of baculovirus-infected insect cells and screened with a specific neutralizing monoclonal antibody. Using this approach, a baculovirus clone was isolated that had markedly increased binding capacity to the monoclonal antibody than the non-modified peptide.

rBV displaying gp64 fusion proteins have also been used successfully for antigen delivery. Lindley *et al.* [30•] describe the development of monoclonal antibodies to the nuclear receptors LXR and FXR following immunization of mice with rBV displaying gp64 amino-terminal LXR and FXR fusion proteins. This methodology provides a novel means of generating and screening for monoclonal antibodies without the need for purified protein.

Baculoviruses as gene delivery vehicles in mammalian cells

Recently, two groups of investigators reported an innovative new use for rBV. Viruses containing mammalian expression cassettes, with transcription of a reporter gene controlled either by the cytomegalovirus (CMV) immediate early promoter [31], or the Rous sarcoma virus promoter [32] can mediate transient gene transfer and expression in mammalian cells, primarily those of hepatic origin. The highest levels of gene expression were seen in primary hepatocyte cultures and cell lines derived from hepatocellular carcinomas, though a low level of reporter gene expression was observed in other cell lines, such as 293, COS-1 and T-47D. Yap *et al.* [33] subsequently demonstrated a somewhat expanded list of cell lines susceptible to baculovirus transduction. These investigators used a chimeric actin–CMV promoter to express T7 RNA polymerase in HeLa, COS7, and porcine kidney cells, in addition to human hepatoma cell lines. The activity of the enzyme was assessed through an indirect method. A follow-up report [34] looked directly at luciferase expression under the control of the same promoter, and in a few additional cell lines. A much larger panel of cell lines was examined by Condreay *et al.* [35••], including cultures of primary human cells. They reported efficient transduction of a wide variety of cell types. Additionally, they found that baculovirus-mediated gene expression directed by the CMV promoter could be significantly enhanced by the addition of butyrate or trichostatin A, a histone deacetylase inhibitor. Another class of viruses that productively infect only insect cells but can mediate recombinant gene expression in mammalian cells are the entomopoxviruses. Derivatives of *Amsacta moorei* entomopoxvirus (AmEPV) have been described that are able to direct the expression of reporter genes under the control of early promoters of the virus in a diverse panel of mammalian cell lines [36]. The viral late promoters were found to be inactive in mammalian cells. This result suggests that some of the early gene products of AmEPV could be expressed in mammalian cells. The transcriptional activity of early promoters of rBV in mammalian cells has not been characterized.

At this point, sufficient data have been reported to support the conclusion that uptake of baculoviruses by mammalian cells is a general phenomenon. A recent report [37] demonstrates that electrostatic interactions are necessary for baculovirus binding to mammalian cells, and that baculoviruses are able to bind to heparin. Although not conclusive, the data suggest that heparan sulfate moieties on the cell surface are involved in binding of the virus. One insight into the mechanism of why certain cells are more efficiently transduced than others is supplied by data from Barsoum *et al.* [38]. They used a virus that had been pseudotyped with the vesicular stomatitis virus G glycoprotein to compare its ability to transduce HeLa cells with an unmodified baculovirus. Their data suggest that the block to efficient gene expression in less susceptible cell lines is not due to a block in entry of the virus into a cell, but is in the ability of the virus to escape intracellular vesicles so that the DNA reaches the nucleus of the cell.

In addition to directing transient expression in mammalian cells, Condreay *et al.* [35••] demonstrated that rBV can also be used to derive stable cells lines. An expression cassette containing a dominant selectable marker was included in the rBV and transduced cells were cultured under antibiotic

selection. Stable derivatives could be obtained at high frequency that maintained expression of the green fluorescent protein reporter gene for multiple passages in culture. They demonstrated that at least 12 kb of the input viral genome was present in a stable Chinese hamster ovary line, though it is not clear if the baculovirus DNA is integrated onto the host cell genome. Another approach towards the same goal was taken by Palombo *et al.* [39]. They constructed a hybrid baculovirus that contained expression cassettes, controlled by mammalian promoters, flanked by the inverted terminal repeats (ITR) of adeno-associated virus (AAV) in order to take advantage of the ability of AAV to integrate its genome into that of its host cell. This virus gives rise to a low frequency of stable colonies of 293 cells, though the frequency can be dramatically increased if the virus is able to direct expression of the AAV *rep* gene product. Furthermore, the ITR-flanked cassettes are integrated into the host genome in the specific site on chromosome 19 that is characteristic of AAV integration.

One stated goal for the construction of the baculovirus–AAV chimera was to develop a novel vector that would be suitable for *in vivo* gene delivery; however, there is mounting evidence that blood components interact with baculovirus to inhibit transfer. Sandig *et al.* [40] made several attempts at *in vivo* delivery of baculovirus vectors to rats and mice by a variety of routes, but saw no evidence of gene transfer. It was demonstrated that fresh serum from various species inactivated baculovirus for gene transfer into hepatoma cell lines, whereas heat-inactivated serum did not. Use of serum deficient in specific complement system factors suggested that the classical pathway of complement was responsible for the inability to effect *in vivo* gene transfer. Further work with depleted sera more conclusively demonstrated involvement of the classical pathway [41•]. Additionally, serum treated with an anti-C5 antibody, or blood and plasma treated with cobra venom factor (to deplete C3) do not fully inactivate the virus, suggesting strategies for enabling the use of baculovirus as an *in vivo* gene therapy vector. An additional strategy using recombinant soluble complement receptor type 1 to inhibit both pathways of the complement cascade, and thus protect baculovirus from inactivation in serum is the subject of a recent report [42]. Another problem with the use of baculoviruses for gene therapy applications is the ability to scale up their purification and concentration. This can be achieved through ultracentrifugation; however, it is difficult to scale up that procedure, and the virus tends to aggregate upon centrifugation. Barsoum [43] describes the use of a cation exchange medium for the concentration of baculoviruses. The virus binds to the resin at the low pH of insect cell medium and is eluted by a shift in pH by the addition of physiological buffer solutions.

The use of baculovirus chimeras to launch the infection of another virus in mammalian cells has been the subject of two reports. This can be a particularly valuable approach to the study of viral replication for viruses that lack a suitable *in vitro* infection model. It is not surprising that the first efforts in this area have been in hepatotropic viruses, as the first reports of baculovirus-mediated gene delivery to mammalian cells suggested that hepatic cells were most susceptible. Delaney and Isom [44••] placed a greater-than-unit length copy of the hepatitis B virus (HBV) genome into a recombinant baculovirus in the anti-sense orientation to the polyhedrin promoter. This construct contains sufficient contiguous HBV sequences to synthesize all of the HBV mRNAs from its endogenous promoters in liver-derived cell lines. Upon transduction of HepG2 cells with the baculovirus–HBV hybrid, high levels of HBV gene products are detected in the cells, and extracellular HBV virions are produced. Fipaldini *et al.* [45•] have exploited baculovirus transduction to study the replication of hepatitis C virus (HCV) by placing the entire HCV cDNA under the control of the CMV promoter in a recombinant baculovirus. Transduction of HuH7 cells with the baculovirus-HCV virus elicited long-term expression of the HCV polyprotein and its correct processing into HCV structural and non-structural gene products. This chimera should prove useful for the analysis of HCV proteins, though it is not yet clear whether HCV virions are produced by this construct.

Conclusions

The diversity of applications of the rBV system continues to expand. The system has proven highly successful for the expression of recombinant proteins in insect cells. It has been shown to be especially useful for the efficient production of functional multi-subunit protein structures, as exemplified by VLPs. Investigations into the co-expression of enzymes involved in protein glycosylsation, folding, cleavage and secretion have demonstrated the potential to significantly enhance the yield of functional recombinant proteins produced by infected insect cells. The application of rBV for the display of foreign proteins and epitopes on the viral surface provides a powerful tool for antigen presentation and the study of protein–protein interactions. The use of rBV, containing mammalian gene regulatory elements, will undoubtedly prove to be a useful tool for gene delivery and expression in mammalian cells. Further investigation into the use of modified rBV for *in vivo* gene delivery may yield additional applications for this versatile expression system.

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