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Genetic Modification of Baculovirus Expression Vectors*

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Abstract: As a protein expression vector, the baculovirus demonstrates many advantages over other vectors. With the development of biotechnology, baculoviral vectors have been genetically modified to facilitate high level expression of heterologous proteins in both insect and mammalian cells. These modifications include utilization of different promoters and signal peptides, deletion or replacement of viral genes for increasing protein secretion, integration of polycistronic expression cassette for producing protein complexes, and baculovirus pseudotyping, promoter accommodation or surface display for enhancing mammalian cell targeting gene delivery. This review summarizes the development and the current state of art of the baculovirus expression system. Further development of baculovirus expression systems will make them even more feasible and accessible for advanced applications.

Key words: Baculovirus; Protein expression; Promoters; Signal peptides; Gene delivery

The baculovirus expression vector system (BEVS) has developed into one of the most attractive methods for expression of recombinant proteins, since the first successful application in the production of human beta interferon[72]. This system exhibits many distinguishing advantages in comparison to others, including high expression level[55], capability of large DNA fragment insertions[15], co- and post-translational modifications like glycosylation, phosphorylation, acylation and amidation[3]. In addition, baculoviruses are able to transduce many kinds of mammalian cells without replicating in those cells[9, 78]. Due to these features, the BEVS is employed not only in producing proteins of interest, but also in surface display[8, 56] and gene therapy[46]. During the last thirty years, the baculovirus has been adapted to function as an efficient expression system, and significant progress has been achieved in pharmaceutical and vaccines applications. Table 1 summarizes the milestones in BEVS development. However, certain limitations remain for BEVS and need to be improved for expanding its application, such as low expression levels of secreted and membrane-bounded proteins[19, 79, 80]. And as the protein processing pathway in insect cell is,

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Table 1. The milestones in the Development of BEVS

Time	Chronicle of Events	Ref.
16th century	The first description of baculovirus disease in Western literature.	(25)
1983	The first successful application of a BEVS in the production of human beta interferon.	(62)
1983	The discovery of the ability of baculoviruses to gain entry into mammalian cells.	(68)
1990	The application of baculoviruses in virus-like particle production.	(67)
1993	The complete DNA Sequence of *Autographa californica* Nuclear Polyhedrosis Virus determined.	(4)
1993	The development of the Bac-to-Bac system.	(49)
1995	The first successful expression of a foreign gene in mammalian cells mediated by AcMNPV.	(28)
1995	Baculovirus vectors used for the production of chimeric, humanized, and human antibodies in insect cells.	(66)
1999	The complete DNA Sequence of *Bombyx mori* nucleopolyhedrovirus determined.	(24)
1999	AcMNPV found to be capable of stimulating antiviral effects in mammalian cell lines.	(27)
2004-2007	BEVS expressed vaccines against *Plasmodium falciparum*, Japanese B encephalitis and human immunodeficiency virus (HIV) were patented.	(34)
2007	The VLPs of human influenza and avian influenza (H5N1) were produced in BEVS.	(10, 52)
2009	The first vaccine produced in BEVS for human use was approved by FDA.	(34)

to a certain degree, different from that of the vertebrate cell, modification of the insect cell pathway is expected[39]. In addition, baculoviruses are lethal pathogens of insects, infected cells end up dying in the last phase of infection, which makes stable protein expression in insect cells infeasible. All these limitations have stimulated investigators to genetically modify baculoviruses for developing better BEVS; as transfer plasmid and genomic DNA of baculovirus are the core components to form recombinant BEVs, they are the major targets for modification.

Baculoviruses are a diverse group of large DNA viruses which are pathogenic for arthropods, especially insects of the order Lepidoptera and are classified into four genera: *Alphabaculovirus*, *Betabaculoviruses Gammabaculovirus* and *Deltabaculovirus*. The virions are enveloped and rod-shaped with a circular double-stranded DNA genome of 80-180 kilo-base-pairs. In the biphasic life cycle of baculoviruses, two morphologically distinct virion phenotypes, the budded virus (BV) and the occlusion-derived virus (ODV), are produced[69]. In cells infected with the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), assembled progeny nucleocapsids begin to egress from the nucleus at about 18 hours post infection. The nucleocapsids then bud through the cytoplasmic membrane to acquire a lipid-containing envelope derived from the plasmalemma and membrane-bound glycoproteins such as GP64, finally become mature BVs, which are capable of secondary infections. In the late phase of pathogenesis, nucleocapsids are reserved in the ring zone of the nucleus, where they are enveloped to form ODVs and are occluded in a paracrystalline matrix called polyhedra. Previous studies indicated that FP25 and EXON0 might be jointly responsible for the BV and ODV nucleocapsid ratio regulation and, ultimately, yield of two kinds of

virions[18].

Transfer plasmids designed to deliver heterologous gene into the baculovirus genome by allelic replacement or transposition are core components for generation of BEV. The first promoter used in a transfer plasmid was derived from polyhedrin, which provided high-level transcription at the very late stage of infection[72]. Since then, many other promoters from viral genes have been engaged in expression of engineered proteins for achieving different purposes[29, 36-38, 75].

In this review, we will concentrate on the genetic modifications of AcMNPV and *Bombyx mori* NPV (BmNPV) expression systems, which are the most commonly used baculovirus expression vectors[51]. The review mainly summarizes the modifications to baculoviruses and the construction of transfer plasmids.

MODIFICATION OF BEVS FOR PROTEIN EXPRESSION IN INSECT CELLS

Promoters and enhancer elements used by BEVS

One significant hallmark of BEVS is their high expression level; however, secreted or membrane-bound proteins are not produced in the anticipated high amounts and are often insoluble and poorly processed in comparison to cytoplasmic or nuclear proteins[19, 44, 45].

The low expression level of secreted proteins may be ascribed to the poor status of the cell due to virus infection. The very late *polh* and *p10* promoters are routinely used to drive exogenous protein expression by which time the cell machinery is expected to have deteriorated[83]. In an attempt to overcome this problem, some earlier promoters were introduced. Hill-Perkins & Possee[29] utilized a promoter from *p6.9*, which encodes a structural protein associated with DNA packaging in the nucleocapsid, to direct the *β*-galactosidase gene. β-galactosidase expression was detected from 8 to 24 hr postinfection. To some extent, they provided an incentre for reseachers to investigate baculovirus vector amelioration to obtain high level production of secretory proteins in earlier stage of insect cells infection. At the same time, a series of recombinant baculovirus vectors were constructed including a promoter from *vp39* and combined promoters from *vp*39 and *polh*. The results indicated that the combination of *vp*39 and *polh* promoters was better for foreign gene expression[75].

Certain baculovirus DNA elements have been shown to be capable of enhancing gene expression and the most frequently applied element is homologous region (*hr*), the origin of viral DNA replication. The introduction of *hr1 of* AcMNPV or *hr3* of BmNPV into the genome, resulted in hyperexpression of reporter genes[13,81]. According to a recent report, the integration of *hr1* into upstream of polyhedrin promoter increased the expression levels of Luciferase, human Protein Kinase B-a and CYP-1A2 by 4.5-, 3.5-, and 3-fold, respectively. The introduction of *hr1* also enhanced the stability of a modified vector through a series of passages[77]. In a BmNPV expression system, a *vp39* promoter enhanced by *hr3* from BmNPV was used; this modification brought larger amounts of correctly folded protein with better activity[36].

In addition, some exogenous promoters were recruited in BEVS, such as human cytomegalovirus immediate-early (CMV-IE) and heat shock 70 (Hsp70) promoters, which could be activated by several baculovirus DNA elements either in insect or

mammalian cells and implement high quality production[48, 82].

Signal peptides used by BEVS

Another possible reason for poor expression level of secretory proteins may due to the heterologous signal peptide that could not be efficiently recognized by the insect cell protein secretory pathway. Therefore, honeybee melittin signal peptide was used and the expression quantity of recombinant protein was increased[74]. Further work was carried out to identify the efficiency of different signal peptides (Table 2). Various insect-derived signal peptides and prosequences were used to express human tissue plasminogen activator in insect cells; however, the expression and secretion levels were not notably improved compared to the native ones[40]. This suggested that the processing signal from other species was not the only impedance to high level production of secretory proteins[34]. However, efforts towards investigating the influence of signal peptides

Table 2. Summary of modifications for baculovirus expression system

Targets	Modifications	Ref.
Baculovirus Genome	Deletion of *cathepsin*	(27,63)
	Deletion of *chitinase*	(57)
	Double deletion of *cathepsin* and *chitinase*	(26,35)
	Triple deletion of *p26,p10* and *p74*	(25)
	BmNPV *fp25k* replaced by AcMNPV *fp25k*	(49)
Insect Promoters	*polh* promoter	(73)
	p10 promoter	(73)
	p6.9 promoter	(24)
	vp39 promoter	(65)
	hybrid *vp39* and *polh* promoter	(65)
	polh promoter enhanced by Homologous Region 1	(67)
	vp39 promoter enhanced by Homologous Region 3	(30)
Mammalian Promoters	Cytomegalovirus(CMV) *ie* promoter	(28)
	Rous sarcoma virus(RSV) promoter	(8)
	SV 40 early promoter	(14)
	WSSV *ie1* promoter	(19)
	CAG promoter	(61)
	murine *ie1* enhancer/promoter upstream of *p10* promoter	(37)
	hybrid CMV promoter and Semliki Forest virus (SFV) replicon	(53)
	hybrid CMV and PDGF β -chain promoter	(41)
Signal Peptides	honeybee melittin signal peptide	(64)
	cecropin B signal peptide	(34)
	Staphylococcal protein A signal peptide	(2)
	human azurocidin signal peptide	(52)
	B.mori bombyxin(*bx*) signal peptide	(54)
	B.mori prophenoloxidase-activating enzyme(*ppae*) signal peptide	(54)
	silkworm SP1 with an additive asparagine in the C-terminal	(18)

from disparate sources on the secretion of proteins are ongoing[2, 60]. The secretion of β1, 3-*N*-acetylglucosaminyltransferase 2 (β3GnT2) fusion protein into the hemolymph of *Bombyx mori* larvae was enhanced when signal peptides of silkworm *B. mori* bombyxin (*bx*) and *B. mori* prophenoloxidase-activating enzyme (*ppae*) were introduced into the BmNPV genome[62]. Recent studies on signal peptide reformation clarified that polar amino acids in the C-terminal region of a signal peptide exerted a strong influence on the secretion of synthesized proteins. Amongst a series of constructed signal peptides derived from silkworm SP1, the one with an added asparagine in the C-terminal region enhanced expression levels of human interleukin IL-4, IL-13, and the extracellular domain of human IL-11 receptor a, each of which harbor a distinct secondary structure. This novel approach for protein secretion improvement was established for the purpose of using in BmN cells and other insect cells[20].

Modification of baculovirus genomes

The work outline in the previous section targeted the transfer vector to improve the yield of secreted proteins. In this section, we discuss modifications to the genome of baculoviruses; these are summarized in Table 2. By modification of baculoviral genomes, metabolic efficiency of insect cells may be improved and the redundancy among baculovirus genes and regulatory circuits could be decreased.

Genes encoding proteins that are detrimental to recombinant protein are common deletion targets. Cathepsin (V-CATH), a cysteine protease encoded by viral gene, prejudices expression levels through proteolysis of recombinant proteins. C*hitinase*, which encodes a secretory protein called chitinase A (ChiA) is another deletion target. ChiA is expressed at the late stages of infection and contains a signal peptide that leads ChiA to the endoplasmic reticulum (ER) where it accumulates due to a KDEL ER retention motif at the carboxy terminus and composes a dense paracrystalline matrix [76]. Both of these examples affect the expression of recombinant secreted and membrane-bound proteins, either through burdening the protein translocation machinery, or obstructing the secretory pathway [31]. The active ChiA plays a crucial role in the proper folding of proV-CATH to form matured V-CATH. Both of ChiA and V-CATH are responsible for liquefaction of the host in the terminal phase of infection but at the cell level, they have a negative impact on recombinant protein expression. The gene encoding V-CATH was also deleted from the AcMNPV and BmNPV for the purpose of reducing the proteolytic degradation of foreign proteins [73].

Along with the technological development, a *v-cath* negative BmNPV bacmid was constructed to improve the production of GFPuv-β1, 3-*N*-acetylglucosaminyltransferase 2 (GGT2) fusion protein, which was confirmed to provide rapid and large scale protein production in silkworms [32]. On the other side, Possee *et al.*,[65] achieved increased expression of secreted proteins with less degradation and enhancing the integration of host cells by removing the *chiA* gene from the genome of AcMNPV. As ChiA and V-CATH are functionally related to each other, a dual deletion AcMNPV bacmid was generated to express the *Theileria parva* sporozoite surface protein p67. The results showed that although there was no major increase in the total yield of the recombinant protein, its hydrolysis was reduced and the integrity of structure was improved[41]. Recently, Hitchman *et al.*,[31]

constructed a *v-cath* and *chiA* dual deletion vector and demonstrated the significantly enhanced output of many secreted and membrane-bound proteins in comparison to the wild type vector.

Other viral genes, which are not essential for virus replication and BV production turn out to be candidates of genetic modification as well and the removal of those genes might improve recombinant protein expression levels and stability. This was confirmed by a study which knocked out *p26, p10 and p74* from a recombinant AcMNPV which was deficient in *chiA* and v-*cath*. Expression levels of EGFP, β-galactosidase (β-gal), heat-shock 90-KD protein 1, alpha (HSPCA1), polo-like kinase 1 (Plk1), phosphatase and tensin homolog (PTEN) and KappaBRas2 were increased to different extents. Indeed, greater stability of p21-activated kinase (PAK5) was detected when expressed by the modified vector[30].

Additional studies on genetic modification of baculovirus genomes to acheive higher expression levels have also been reported. A lepidopteran baculovirus-specific gene, *fp25K*, is involved in the few-polyhedron (FP) phenotype during virus passage in cultured cell lines[28]. The mutation of *fp25K* in AcMNPV resulted in a decreased number of ODVs and an increase in the production of budded virus, while the deletion of *fp25K* in BmNPV led to lower BV yield. When *fp25K* of BmNPV was replaced with the corresponding gene in AcMNPV, the results illustrated that production of ODV and BV were improved remarkably. This finding was applied in the optimization of a BmNPV-based expression vector, and the new vector with AcMNPV *fp25K* is capable of increasing the expression of recombinant proteins through enhancing the activity of the *polh* promoter[57].

Nevertheless, there is still enormous potential for modification of the baculovirus genome to improve the amount of secreted proteins. The baculovirus possesses a large genome and genes that are nonessential or even a burden to propagation of virus in cell culture could be deleted for the purpose of improving baculovirus expression. More research is needed on this subject.

Modification of BEVS for expression of protein complexes

Baculoviruses are also employed in the expression of protein complexes in insect cells. Traditionally, multiple genes were expressed in the same cell through co-infection of several viruses, each containing one single exogenous gene in its genome. However, this method is rather inefficient to achieve high level of production.

Novel approaches tend to genetically modify the vector by cloning multiple genes into one virus, which provide an enhanced yield. For example, co-expression transfer vectors such as pAcUW51, pAcAB4 and pFastBac-DUAL were created to generate recombinant baculoviruses. Functional expression of the human TAP complex (comprised of two proteins) was detected in Sf9 cells infected by a recombinant AcMNPV which could express two proteins at the same time[54]. Also, rotavirus 2/6-virus-like particles (VLP) produced by a dual baculovirus expression vector demonstrated high level of immunogenicity and protective efficacy[7]. Another bi-cistronic baculovirus expression vector was constructed by insertion of the Rhopalosiphum padi virus 5' internal ribosome entry site in between two expression cassettes[14]. New transfer vectors pUCDM and pFBDM with a multiplication module in the

middle of two expression cassettes were constructed to avoid integration of foreign gene through restriction enzyme cutting in the process of multiple protein complex production[6]. In another approach, Noad *et al.* identified 7 loci in the AcMNPV genome other than *polyhedrin* and *p10* that were suitable for high level production of heterologous proteins. Two protein-, four protein- and eight protein- complexes including virus-like particles and cellular chaperone complexes were successfully produced through iterative modification of bacmid DNA[58].

MODIFICATION OF BEVS FOR GENE DELIVERY

The earliest reports of baculovirus entry into mammalian cells can be found in the literature from the 1980s'[26, 78]. Tjia *et al.* first found that AcMNPV was able to enter mammalian cells such as Human HeLa cells, primary human embryonic kidney cells, simian CV1 cells, hamster BHK21(B3) cells and Muntiacus muntjak cells, although they failed to replicate or transcribe in these cells[78]. Afterwards, many other nonpermissive cell lines, such as CP 169 and CHO-K1, were verified to have the ability to adsorb viral DNA when inoculated with AcMNPV[26].

Promoters used for expression of exogenous gene in mammalian cells

While the evidence indicated that baculoviruses held great promise as a novel non-human viral DNA vector for gene delivery into mammalian cells, as an alternative to traditional 'vehicles' such as retroviruses or adenoviruses. The key problem was how to increasing the expression of foreign gene in mammalian cells. Carbonell *et al.* proposed that the type of promoter used to drive gene transcription was very crucial for baculovirus mediated gene expression in mammalian cells[11]. However, an early study of a modified AcMNPV containing the Rous sarcoma virus (RSV) promoter failed to produce detectable chloramphenicol acetyltransferase (CAT) in A549 human lung carcinoma cells or in L929 mouse cells. This may have been due to some impedance to the entry or expression of AcMNPV in mammalian cells, or could be the consequence of low multiplicity of transduction[12]. In any case, subsequent studies focused on modifying the vector with promoters which were constitutive in mammalian hosts (Table 2). The *Luciferase* gene was successfully expressed at high level under the control of the CMV-IE promoter in hepatocytes mediated by AcMNPV, which confirmed that the vector could express an exogenous gene in mammalian cells, under the conditions that the gene was driven by promoter known to be active in mammalian cells[33]. In another case, AcMNPV was showed to infect human hepatoblastoma cell line HepG2 and rat primary hepatocytes, and express the reporter gene *Lacz* under the control of a Rous sarcoma virus (RSV) promoter[9]. Alternative mammalian promoters included the CAG promoter, a composite promoter consisting of the CMV IE enhancer, the chicken β-actin promoter and the rabbit β-globin polyadenylation signal, as well as the SV40 early promoter were utilized[16, 71]. Different foreign genes were transiently expressed *in vitro* and *in vivo* under the control of the promoters mentioned above in hepatocytes and other mammalian cell lines such as neural cells, human hepatoma cells (HuH-7), human keratinocyte cells, rabbit aortic smooth muscle cells and pancreatic islet cells[1, 16, 50, 70, 71].

In addition, some shuttle or hybrid promoters were

inserted into a genetically modified baculovirus to facilitate the application of baculovirus-mediated gene delivery. The white spot syndrome virus (WSSV) ie1 promoter is one such promoter, which functions both in insect and mammalian cells and demonstrates a higher activity than the baculovirus early-to-late (ETL) promoter in several mammalian cell lines[21]. A new genetically engineered vector harboring a compound promoter, which consists of human and murine cytomegalovirus immediate-early enhancer/promoter elements and the baculoviral *polh* and *p10* promoter was constructed for simultaneous expression in vertebrate and insect cells. Highest expression efficiency in vertebrate cells was achieved when the murine *ie1* enhancer/promoter was upstream of the baculoviral *p10* promoter[43]. Another recombinant baculovirus, which possessed a hybrid promoter with a CMV promoter and a Semliki Forest virus (SFV) replicon, demonstrated high transduction efficiency and high-level expression of reporter protein (EGFP) in mammalian cells[61]. Hybrid promoters also benefit specific delivery. The enhancer of human CMV IE promoter fused to the human platelet-derived growth factor (PDGF) β-chain promoter constituted an enhanced neurone-specific promoter to provide cell type-specific transgene expression[47].

Pseudotyping baculovirus for gene delivery

Improvement of transduction efficiency and cell-specific gene delivery were achieved through baculovirus pseudotyping. In the first instance, Barsoum *et al.* constructed a modified baculovirus, which presented glycoprotein G from VSV on its envelope. This novel vector obtained a transduction efficiency 10-fold greater than one lacking VSV G[5]. This vector was used in combination with human AFP (α-fetoprotein) promoter for hepatocyte-specific gene expression[63]. The application of the pseudotype baculovirus *in vivo* was also studied and the expression level of reporter protein was significantly increased in myoblasts, myotubes and quadriceps of infected mice[64]. Five years later, Kaikkonen *et al.* demonstrated that the 21-amino-acid ectodomain with transmembrane and cytoplasmic tail domains of VSV-G (VSV-GED) was functional enough to improve gene delivery efficiency of the pseudotype baculovirus[42].

Surface display for targeted gene delivery

Recombinant baculoviruses displaying specific ligand binding moieties are effective for enhanced and targeted gene delivery. A single chain antibody fragment (scFv) specific for the carcinoembryonic antigen (CEA) or the synthetic IgG binding domains (ZZ) derived from protein A of *Staphylococcus aureus* fused with the N-terminus of the baculoviral gp64 protein were displayed on the surface of AcMNPV for specific gene transfer to CEA expressing cells (PC-3) and BHK cells, respectively. Expression of EGFP was observed in both of the transduced cell lines[59]. Similarly, another approach based on a high-affinity receptor–ligand pair successfully enhanced transduction through displaying avidin on the viral surface. Furthermore, EGF receptor-expressing (SKOV-3) cells demonstrated a higher efficiency for accepting engineered vector with biotinylated epidermal growth factor (EGF) on the surface[68]. The RGD motifs within the C-terminus of coxsackie virus A9 and human parechovirus 1 VP1 proteins, or comprising 23 amino acids from the foot-and-mouth disease virus (FMDV) VP1, were displayed on the viral surface to improve transduction of mammalian cells[17, 53]. Finally, a baculovirus with a short peptide

motif from gp350/220 of Epstein–Barr virus proved to be a B cell-specific vector targeting the CD21 protein on the surface of B-lymphocytes[22].

Modification of baculovirus for *in vivo* gene delivery

The fact that modified baculovirus for *in vivo* gene therapy could be inactivated by complement system hinders the wider application of the vector. Efforts were therefore directed towards overcoming this bottleneck by generating a complement-resistant baculovirus. In order to enhance the efficiency of transductions, a genetically engineered baculovirus displaying the human decay accelerating factor gene (DAF) was generated. DAF is a complement-regulatory factor which blocks the complement system at the central step of both classical and alternative pathways by increasing rate of decay of C3bBb and C4b2a complexes, thus enhancing the efficiency of transduction. The DAF complement-resistant baculoviruses have been proved to be stable in complement-sufficient neonatal rats[35].

CONCLUSION

There is no doubt that baculovirus expression vectors are highly attractive and powerful tools for expression of heterogenous proteins in both insect and mammalian cells[46]. Thousands of proteins and multiple protein complexes have been successfully produced through the application of recombinant baculovirus expression systems. Baculoviruses have also emerged as a useful gene delivery vehicle for gene therapy[23]. Remarkable achievements have been made via genetic modifications with the purpose of improving the vector, these are summarized in Table 2 in this review. Further development of baculovirus expression systems will make them even more feasible and accessible for advanced applications including human pharmaceuticals and vaccines.

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