Unit Title

Running Title: Overview of the Baculovirus Expression System

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Significance Statement

The baculovirus expression vector system (BEVS) is widely used for recombinant protein production throughout the world. The system is based on gene expression in virus-infected insect cells that have the capability to recognise and process translational modification sites, allowing the formation of authentic eukaryotic proteins. The BEVS offers other advantages such as inherent safety, scalability and speed. These features combined have led to wide-spread use including production of vaccine targets in both human and veterinary medicine.

Words = 76

ABSTRACT

This chapter provides information on the replication cycle of insect baculovirus to provide an understanding of how this virus has been adapted for use as an expression vector for recombinant proteins in insect cells. We provide an overview of the virus structure and its unique bi-phasic replication cycle that has been exploited in developing the virus as an expression vector. We also review the development of the baculovirus expression vector system (BEVS) since the mid 1980's to the present day in which the BEVS is now an established tool for the production of a range of recombinant proteins and multi-protein complexes including virus-like particles. We describe advances made to the BEVS to allow the rapid and easy production of recombinant viruses and developments to improve protein

yield. We finish by describing the application of recombinant BacMam as vectors for the delivery of genes into mammalian and human cells.

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Keywords: Baculovirus expression vector, BEVS, BacMam, insect cells

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INTRODUCTION

Baculoviruses are arthropod-specific viruses that belong to the family *Baculoviridae* and infect more than 600 host species found in a number of different orders (reviewed by Herniou *et al.*, 2003; Possee *et al.*, 2010). In nature, baculoviruses play a key role in regulating insect populations and so have successfully been used as pesticides due to their host specificity and safety for the environment and human health (reviewed by Bonning & Hammock, 1992; van Beek & Davis, 2016).

An increase in the biological understanding of the prototype baculovirus, Autographa californica multiple nucleopolyhedrovirus (AcMNPV), lead to the application of baculoviruses as an expression system in the mid 1980's (Smith *et al.*, 1983a). Since then the baculovirus expression vector system (BEVS) has been used to express thousands of recombinant targets and some of these have been utilized for the generation of commercially available vaccines (reviewed by Felberbaum, 2015). The BEVS proved highly suitable for recombinant protein production due to the high level of gene expression driven from a very late gene promoter, the polyhedron gene promoter, in virus-infected insect cells.

The insect cells used in the BEVS have the capability to recognise and process signal peptides, support oligomerisation and undertake post-translational modifications such as glycosylation, acylation, phosphorylation and disulphide bond formation, enabling the correct formation of authentic eukaryotic proteins; in contrast to prokaryotic expression systems (Geisler & Jarvis, 2009; Klenk, 1996). Another advantage of the BEVS is that the insect cells grow in serum-free media and can easily be scaled up in shaking cultures or bioreactors at 27-28°C.

This unit describes the variety of different BEVS and their advantages and disadvantages; protocols for using the different systems can be obtained from the appropriate suppliers. However, protocols related to the generation of baculovirus vectors using the homologous recombination method will be discussed further in UNIT 5.5, along with more general protocols for expression optimisation and large scale production.

BACULOVIRUS LIFE CYCLE

The common rod-shaped nucleocapsid of baculoviruses varies between 200-400 nm in length, is about 36 nm wide and encloses a double-stranded, circular supercoiled DNA genome, which is between 80-180 kbp in size (Funk *et al.*, 1997). The prototype AcMNPV, which forms the basis of most expression vectors, was the first baculovirus to be sequenced completely and was found to be 134kbp in size and harbours 154 ORFs (Ayres *et al.*, 1994).

Baculoviruses have a bi-phasic replication cycle, which is driven by the two structurally distinct forms of the virus; budded virus (BV) and the occlusion-derived virus (ODV). Primary infection and horizontal transmission of baculovirus in host larvae is mediated by the oral route using ODV, after ingestion of occlusion bodies (OBs) (Figure 5.4.1). Secondary infection involving the spread of infection from tissue to tissue within the host larvae is initiated by the BV form (reviewed by Possee *et al.*, 2010). Budded virus is also the form that is used in cell culture for the BEVS, as it has been shown to be about 1,800-fold more infectious than ODV (Volkman *et al.*, 1976). Virus gene expression, DNA replication and the synthesis of structural proteins occurs during four successive phases: immediate-early, delayed-early, late and very late (reviewed by Slack & Arif, 2007). New progeny virus form in the nucleus and in the earlier stages of infection, nucleocapsids leave the nucleus to migrate through the cytoplasm to the plasma membrane to form BV, whereas in the very late stages, they remain in the nucleus and become occluded as ODV (reviewed by Slack & Arif, 2007).

The BV obtains its envelope when the nucleocapsids bud through the cell plasma membrane. The major envelope protein that is required for BV attachment, fusion and budding is acquired during the budding stage, and for AcMNPV this protein is GP64 (reviewed by Slack & Arif, 2007). Nucleocapsids destined to become ODV remain in the nucleus and gain an envelope by an unknown process that may be *de novo* synthesis or from the nuclear membrane. The ODV become occluded in large proteinaceous OBs, which in the case of AcMNPV is formed from polyhedrin protein, which is expressed to very high levels in the very late stage of infection. The OBs allow the ODV to remain viable in the environment until a susceptible host is present, which can be several seasons. For a more comprehensive review of baculovirus life cycle, see Possee *et al.* (2010) or Slack and Arif (2007).

The polyhedrin gene along with the other highly expressed very late gene, *p10*, both produce proteins that are only required for horizontal transmission and not for systemic infection within the larva or for the spread of infection in insect cell culture; these proteins are, therefore, not essential for BV production (Smith *et al.*, 1983b; Summers, 2006; Vlak *et al.*, 1988). Therefore, the polyhedrin or *p10* gene coding regions can be replaced by recombinant genes, retaining the very strong promoters to drive foreign gene expression.

BACULOVIRUS EXPRESSION SYSTEM

Human β-interferon was the first protein successfully produced to high levels using baculovirus vectors, when its gene coding region was inserted in place of the polyhedron gene (*polh)* coding region in the AcMNPV genome (Smith *et al.*, 1983a). There have been several advances in the BEVS since the mid 1980's, which are explained in the following sections. Due to the large size of the baculovirus genome, it is difficult to insert genes directly into the virus genome using molecular cloning techniques, although there is one commercial system that uses this approach (BaculoDirect™, Thermo Fisher Scientific Inc). Most baculovirus expression systems involve the use of an intermediate plasmid vector into which the foreign gene is cloned, and then various methods have been developed to transfer the gene from the plasmid vector into the virus genome, as described below (reviewed by King *et al.*, 2007).

Homologous recombination in insect cells

The expression systems classified in this section produce recombinant baculovirus in insect cells by homologous recombination between the baculovirus genome and the transfer plasmid containing the gene of interest, which is normally under of the control of the *polh* promoter. However, alterative transfer plasmids exist that contain other insect promoters (*p10*, *p6.9*) and those that allow simultaneous expression of two target genes (Weyer & Possee, 1991) or more (Belyaev & Roy, 1993). To facilitate recombination, the gene of interest in the transfer vector is flanked by fragments of the baculovirus genome identical to the sequences up and downstream of the desired insertion site, generally the *polh* locus.

The original recombinant baculoviruses were produced by homologous recombination between the intact, circular AcMNPV genome and a transfer vector and resulted in the production of recombinant, polyhedrin-negative viruses. However, because homologous recombination occurs at very low frequency, only small numbers of recombinant viruses were generated in a background of polyhedrin-positive (parental) viruses. The polyhedrin-negative viruses had to be isolated via several rounds of tedious plaque purification in which virus-infected cells containing a polyhedra-negative phenotype were identified by light microscopy (King & Possee, 1992; Smith *et al.*, 1983a).

This original method was first improved by linearizing the AcMNPV genome at the insertion site (*polh* locus) before the homologous recombination step. This resulted in a greater recovery of recombinant viruses (~30%) because linearised virus DNA cannot replicate and produce infectious virus (Kitts *et al.*, 1990). The system was improved further by the generation of a triple *Bsu361*-disgested AcMNPV genome that contained *lacZ* at the *polh* locus, which was termed BacPAK6 (Kitts & Possee, 1993). The triple-digested and linearized BacPAK6 DNA lacked a functional *orf1629* that is essential for BV production; this gene is restored after homologous recombination with the transfer plasmid that contains *orf1629* in the flanking sequences. This development greatly decreased the parental virus background,

improving the efficiency of recombinant virus production up to 90% and reduced the number of plaque purification rounds required to isolate a recombinant virus. Replacement of the native *polh* coding sequence with that of *lacZ* also enabled plaques containing recombinant virus to be identified by blue-white selection, with parental non-recombinant virus plaques turning blue in the presence of X-gal (Kitts & Possee, 1993). The BacPAK6 system formed the basis of a number of commercial baculovirus systems in the 1990s and is currently available from Oxford Expression Technologies (OET, www. oetltd.com).

A new platform technology that built on the BacPAK6 system has been developed that allows a rapid one-step generation of recombinant viruses, and which is therefore suitable for high throughput production (Possee *et al.*, 2008). This platform is based on maintaining an AcMNPV genome that contains only part of *orf1629* in *E. coli*, due to the insertion of a bacterial artificial chromosome (BAC) at the *polh* locus. Therefore, this removes the requirement for DNA linearization prior to the homologous recombination step in insect cells and results in 100% recovery of recombinant virus. There is therefore no need for time-consuming plaque-purification steps, reducing the time and complexity of making recombinant baculoviruses. The system has been commercialised as the *flashBAC™* system (Possee *et al.*, 2008) by OET Ltd

The yield and quality of recombinant protein has been improved by making non-essential gene deletions in the virus genomes that are used in the *flash*BAC™ system. This has resulted in the two new categories of expression vector namely *flash*BACGOLD™ (FBG; in which chitinase and cathepsin have been deleted) (Hitchman *et al.*, 2010b) and *flash*BACULTRA™ (FBU; in which chitinase, cathepsin, *p26*, *p10* and *p74* have been deleted) (Hitchman *et al.*, 2010a). Deletion of chitinase, which is targeted to and partially blocks the endoplasmic reticulum in infected cells, results in improved yields of membrane-targeted or secreted proteins (Hitchman *et al*., 2010b). Deletion of cathepsin improves the yield of proteins that might otherwise be degraded by this viral proteinase (Hitchman *et al*., 2010b).

Site-directed transposition in *Escherichia coli*

A major improvement, to avoid the work involved in the isolation of recombinant viruses by plaque purification, was the generation of an AcMNPV bacmid that can be amplified and modified in *E.coli* but also is infectious in insect cells (Luckow *et al.*, 1993). This bacmid contains *lacZ* as a selection marker and Tn7 transposition sites. The other major element of this system is a transfer vector that contains the gene of interest flanked by Tn7 transposition sites. When the transfer vector is introduced into *E.coli* containing the AcMNPV bacmid, a site-directed transposition occurs between the bacmid genome and the transfer vector, inserting the gene of interest into the *polh* locus disrupting the *lacZ* sequence. Once the recombinant bacmid is identified, based on antibiotic resistance and blue/white selection on agar plates, it is amplified, isolated and introduced into insect cells

for BV production (Luckow *et al.*, 1993). Although this system removes the requirement of plaque-purification, there are still several steps necessary in *E. coli* before recombinant viruses can be generated in insect cells. This system has been designated as Bac-to-Bac® and is commercially available from Thermo Fisher Scientific Inc.

A second baculovirus expression system using a similar technique of Tn7 transposition in *E. coli* has been designed to allow the production of multiprotein complexes. This system allows the production of a recombinant baculovirus that can contain multiple target genes that are under the control of either the *p10* or *polh* promoters. Each gene of interest requires cloning into a transfer vector using standard cloning techniques. The assembly of the multigene expression transfer vector is aided by homologous and site-specific recombinases. Interestingly, this system offers alternative method for construction of a multigene expression transfer vector that could in theory contain an unlimited number of target genes. This involves using standard cloning techniques that allows specific restriction sites to be recycled after each insertion into the transfer vector. This system is designated as MultiBac™ and is commercially available from Geneva Biotech (Berger *et al.*, 2004; Fitzgerald *et al.*, 2006).

A potential drawback to these multi-bacmid based systems is the challenge of isolating recombinants from the parental bacmid, but the development of the BVBoost system improved the recovery of recombinant bacmids at the selection stage by the addition of the negative-selection marker, *sacB* (Airenne *et al.*, 2003).

BacMam vectors

The BEVS has been further developed beyond recombinant protein expression in insect cells and into the delivery of target genes into mammalian cells, for protein production and for gene therapy purposes. In this development virus vectors are refered to as BacMam and the recombinant baculovirus contains an expression cassette with a mammalian cell or virus promoter, replacing the *polh* or other baculovirus promoter. The recombinant BacMam enters mammalian cells by transduction, allowing the delivery of the expression cassette, but not the replication of the baculovirus as insect promoters are not active in mammalian cells (reviewed by Airenne *et al.*, 2013; Kost *et al.*, 2005). Recombinant BacMam can be made by any of the baculovirus technologies described above, as long as the transfer vector contains the appropriate promoter, which is most commonly the CMV early gene promoter. This approach has recently been shown to work *ex vivo*, where a BacMam transduction was successfully used to deliver a target gene to a whole porcine kidney (Hitchman *et al.*, 2016).

LITERATURE CITED

Airenne, K. J., Hu, Y. C., Kost, T. A., Smith, R. H., Kotin, R. M., Ono, C., Yla-Herttuala, S. (2013). Baculovirus: an insect-derived vector for diverse gene transfer applications. *Mol Ther, 21*(4), 739-749.

Airenne, K. J., Peltomaa, E., Hytonen, V. P., Laitinen, O. H., & Yla-Herttuala, S. (2003). Improved generation of recombinant baculovirus genomes in Escherichia coli. *Nucleic Acids Res, 31*(17), e101.

Ayres, M. D., Howard, S. C., Kuzio, J., Lopez-Ferber, M., & Possee, R. D. (1994). The complete DNA sequence of Autographa californica nuclear polyhedrosis virus. *Virology, 202*(2), 586-605.

Belyaev, A. S., & Roy, P. (1993). Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells. Nucleic Acids Res, 21(5), 1219-1223.

Berger, I., Fitzgerald, D. J., & Richmond, T. J. (2004). Baculovirus expression system for heterologous multiprotein complexes. *Nat Biotechnol, 22*(12), 1583-1587.

Bonning, B. C., & Hammock, B. D. (1992). Development and potential of genetically engineered viral insecticides. *Biotechnol Genet Eng Rev, 10*, 455-489.

Felberbaum, R. S. (2015). The baculovirus expression vector system: A commercial manufacturing platform for viral vaccines and gene therapy vectors. *Biotechnol J, 10*(5), 702-714.

Fitzgerald, D. J., Berger, P., Schaffitzel, C., Yamada, K., Richmond, T. J., & Berger, I. (2006). Protein complex expression by using multigene baculoviral vectors. *Nat Methods, 3*(12), 1021-1032.

Funk, C. J., Braunagel, S. C., & Rohrmann, G. F. (1997). Baculovirus structure. In L. K. Miller (Ed.), The Baculoviruses (pp. 7-27). New York: Plenum Press.

Geisler, C., & Jarvis, D. (2009). Insect cell glycosylation patterns in the context of biopharmaceuticals. *Post-translational Modification of Protein Biopharmaceuticals*, 165-191.

Herniou, E. A., Olszewski, J. A., Cory, J. S., & O'Reilly, D. R. (2003). The genome sequence and evolution of baculoviruses. *Annu Rev Entomol, 48*, 211-234.

Hitchman, E., Hitchman, R. B., & King, L. A. (2016). BacMam Delivery of a Protective Gene to Reduce Renal Ischemia-Reperfusion Injury. *Hum Gene Ther*.

Hitchman, R. B., Possee, R. D., Crombie, A. T., Chambers, A., Ho, K., Siaterli, E., King, L. A. (2010a). Genetic modification of a baculovirus vector for increased expression in insect cells. *Cell Biol Toxicol, 26*(1), 57-68.

Hitchman, R. B., Possee, R. D., Siaterli, E., Richards, K. S., Clayton, A. J., Bird, L. E., King, L. A. (2010b). Improved expression of secreted and membrane-targeted proteins in insect cells. *Biotechnol Appl Biochem, 56*(3), 85-93.

King, L. A., Hitchman, R., & Possee, R. D. (2007). Recombinant baculovirus isolation. *Methods Mol Biol, 388*, 77-94.

King, L. A., & Possee, R. D. (1992). *The Baculovirus Expression System. A Laboratory Guide* (First ed.). London: Chapman & Hall.

Kitts, P. A., Ayres, M. D., & Possee, R. D. (1990). Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic Acids Res, 18*(19), 5667-5672.

Kitts, P. A., & Possee, R. D. (1993). A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques, 14*(5), 810-817.

Klenk, H.-D. (1996). Post-translational modifications in insect cells. In *Insect Cell Culture: Fundamental and Applied Aspects* (pp. 139-144): Springer.

Kost, T. A., Condreay, J. P., & Jarvis, D. L. (2005). Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat Biotechnol, 23*(5), 567-575.

Luckow, V. A., Lee, S. C., Barry, G. F., & Olins, P. O. (1993). Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in Escherichia coli. *J Virol, 67*(8), 4566-4579.

Possee, R. D., Griffiths, C. M., Hitchman, R. B., Chambers, A., Murguia-Meca, F., Danquah, J., King, L. A. (2010). Baculoviruses: biology, replication and exploitation. In: Asgari, S and Johnson, K Insect Virology. Great Britain: Caister Academic Press. p35-58.

Possee, R. D., Hitchman, R. B., Richards, K. S., Mann, S. G., Siaterli, E., Nixon, C. P., King, L. A. (2008). Generation of baculovirus vectors for the high-throughput production of proteins in insect cells. *Biotechnol Bioeng, 101*(6), 1115-1122.

Slack, J., & Arif, B. M. (2007). The baculoviruses occlusion-derived virus: virion structure and function. *Adv Virus Res, 69*, 99-165. doi:10.1016/S0065-3527(06)69003-9

Smith, G. E., Summers, M. D., & Fraser, M. J. (1983a). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol Cell Biol, 3*(12), 2156-2165.

Smith, G. E., Vlak, J. M., & Summers, M. D. (1983b). Physical Analysis of Autographa californica Nuclear Polyhedrosis Virus Transcripts for Polyhedrin and 10,000-Molecular-Weight Protein. *J Virol, 45*(1), 215-225.

Summers, M. D. (2006). Milestones leading to the genetic engineering of baculoviruses as expression vector systems and viral pesticides. *Adv Virus Res, 68*, 3-73.

van Beek, N., & Davis, D. C. (2016). Baculovirus Insecticide Production in Insect Larvae. *Methods Mol Biol, 1350*, 393-405. doi:10.1007/978-1-4939-3043-2_20

Vlak, J. M., Klinkenberg, F. A., Zaal, K. J., Usmany, M., Klinge-Roode, E. C., Geervliet, J. B., van Lent, J. W. (1988). Functional studies on the p10 gene of Autographa californica nuclear polyhedrosis virus using a recombinant expressing a p10-beta-galactosidase fusion gene. *J Gen Virol, 69 (Pt 4)*, 765-776. doi:10.1099/0022-1317-69-4-765

Volkman, L. E., Summers, M. D., & Hsieh, C. H. (1976). Occluded and nonoccluded nuclear polyhedrosis virus grown in Trichoplusia ni: comparative neutralization comparative infectivity, and in vitro growth studies. *J Virol, 19*(3), 820-832.

Weyer, U., & Possee, R. D. (1991). A baculovirus dual expression vector derived from the Autographa californica nuclear polyhedrosis virus polyhedrin and p10 promoters: co-expression of two influenza virus genes in insect cells. *J Gen Virol, 72 (Pt 12)*, 2967-2974. doi:10.1099/0022-1317-72-12-2967

FIGURE LEGENDS

Figure 5.4.1 The baculovirus replication cycle

Schematic representation of the Baculovirus replication cycle

INGESTION: The initial infection occurs when a larva inadvertently consumes occlusion bodies (OBs), which travel to the larva midgut. The alkaline environment (pH 10-11) of the midgut causes the OBs to dissolve, releasing the occlusion-derived viruses (ODVs). **ENTRY TO THE MIDGUT**: The ODVs cross a physical barrier (peritrophic membrane) to access the columnar cells, where they enter through direct membrane fusion to the microvillus. The nucleocapsid is released into the cytosol and migrates to the nucleus to deliver the viral genome. This results in a cascade of gene expression where viral genes are expressed, structural genes are synthesized, DNA replication begins and new progeny viruses are assembled and released. **BUDDED VIRUS:** The BV production occurs after the transcription of late genes between six to 24 hours and is involved in spreading the infection throughout the larva host. The BV enter the cell through receptor mediated endocytoisis. As the endosome acidifies, virus and endosomal envelopes fuse, releasing nucleocapsids into the cytoplasm. The nucleocapsids migrate to the nucleus and release the viral genome. The virus

replication cycle is initiated as described earlier. **OCCLUSION BODIES:** During the very late phase (18 hours onwards), nucleocapsids are retained in the nucleus, acquire envelopes to form ODVs, and are embedded within a crystalline matrix of polyhedrin to form OBs. Mature OBs are released by cell lysis and their stability in the environment allows them to persist until a susceptible host is once again present.