Thirty years of baculovirus–insect cell protein expression: from dark horse to mainstream technology

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In December 1983, a seminal paper appeared on the overexpression of human IFN-β in insect cells with a genetically engineered baculovirus. The finding that baculoviruses produced massive amounts of two proteins (polyhedrin and p10) by means of two very strong promoters and that the corresponding genes were dispensable for virus propagation in insect cells was crucial in the development of this expression system. During the next 30 years, major improvements were achieved over the original baculovirus expression vector (BEV) system, facilitating the engineering of the baculovirus vectors, the modification of the sugar moieties of glycoproteins expressed in insect cells and the scale-up of the cell culture process. To date, thousands of recombinant proteins have been produced in this successful expression system, including several protein-based human and veterinary vaccines that are currently on the market. Viral vectors based on adeno-associated virus are being produced using recombinant baculovirus technology and the first gene therapy treatment based on this method has been registered. Specially adapted BEVs are used to deliver and express heterologous genes in mammalian cells, and they may be used for gene therapy and cancer treatment in the future. The purpose of this review is to highlight the thirtieth ‘anniversary’ of this expression system by summarizing the fundamental research and major technological advances that allowed its development, whilst noting challenges for further improvements.

Introduction

The first paper that illustrated the potential of using the baculovirus Autographa californica multiple nucleopolyhedrovirus (AcMNPV) to produce recombinant proteins in insect cells was published by the group of Max Summers at Texas A&M University in December 1983 (Smith et al., 1983b). In that seminal paper, Smith et al. (1983b) described the expression of human IFN-β with a recombinant AcMNPV virus by exploiting the polyhedrin promoter. Only a few months later, a similar paper was published by Lois Miller and co-workers (at the time at the University of Idaho), in this case on the expression of Escherichia coli β-galactosidase (Pennock et al., 1984). These papers hallmark the birth of the baculovirus expression vector (BEV) system. During the 30 years since these publications, baculovirus-based expression technology has matured considerably, and is now commonly used to produce proteins of scientific interest and to manufacture commercial vaccines worldwide, both for human and veterinarian use. In addition, recombinant baculoviruses have found applications as gene delivery vectors for mammalian cells and as expression vectors for adeno-associated virus (AAV)-based gene therapy products.

Here, we celebrate the thirtieth anniversary of the BEV system by briefly reviewing its development, and giving credit to its pioneers and major contributors. Today, this technology serves numerous purposes for scientific research, and in veterinary and human medical applications. We also present a forward view on what more may be possible to further develop and optimize this protein production system. We refer to other review papers with more detailed information to allow researchers to work with this system (e.g. Contreras-Gómez et al., 2014; Jarvis, 2009; Possee & King, 2007; Rohrmann, 2013; van Oers, 2011).

Preceding baculovirus research

The pioneering fundamental research that allowed the first use of AcMNPV as an expression vector is described in an excellent review by Summers (2006). Here, we present a short history summarizing the milestones in knowledge gain and technological development (Table 1) that enabled successful expression of heterologous genes in insect cells using a baculovirus vector. It all started with the establishment of insect cell lines in specially developed insect cell culture media (Gaw et al., 1959; Grace, 1962; Hink, 1970). This achievement was followed by the initial discovery and biological analysis of the AcMNPV virus, isolated by D. L. Clancy from a single alfalfa looper (A. californica) specimen (Vail et al., 1971). The observation that the haemolymph of
Molecular characterization of the polyhedrin gene (polh) helped in the development of the Baculovirus Expression System (BEV) technology and user convenience.

MNPV-infected caterpillars were highly infectious for cultured insect cells (Vaughn & Faulkner, 1963) and allowed for the molecular analysis of DNA of plaque-isolated natural and recombinant genotypes. Restriction enzyme analysis to the HindIII fragment V (Adang & Miller, 1982). The sequence of the polh gene was published in 1983 (Hooft van Iddekinge et al., 1983).

Experimental evidence showing that the polyhedrin protein was not essential for virus replication in cell culture (Smith et al., 1983a) was a key finding and fundamental to BEV design. Smith et al. (1983a) generated the evidence in co-transfection experiments using purified viral DNA and plasmid vectors that carried the above-mentioned AcMNPV EcoRI fragment I from which various parts of the polh gene had been deleted. The authors were able to obtain infectious viruses that they selected based on the OB- phenotype of infected-cell plaques (Fig. 1b).

First BEVs

In the same year, Smith et al. (1983b) published their data on the expression of human IFN-β with a recombinant baculovirus. They used plasmids that carried the coding sequence of IFN-β together with its 3’ UTR harbouring the polyadenylation motif for their co-transfection experiments. The IFN-β sequence was inserted at several locations in the AcMNPV EcoRI fragment I in the proximity of the putative polh promoter. Cells infected with the purified, OB- recombinant virus produced high levels of IFN-β, of which 95% was secreted into the culture medium. The authors also demonstrated that the IFN-β signal peptide was cleaved off and that the recombinant IFN-β was glycosylated, indicating that it followed the normal protein export route via the endoplasmic reticulum (ER). The choice of the heterologous gene suggests that the authors realized the potential impact of their finding as they produced a protein of medical interest. A few years after the initial development of the BEV system, AcMNPV was used to produce another protein of medical interest – human IL-2 (Smith et al., 1985). A similar procedure was followed to express human IFN-α in silkworm cells, using the baculovirus Bombyx mori nucleopolyhedrovirus (BmNPV) (Maeda et al., 1985).

As early as 1983–1984, several properties of AcMNPV were recognized as being helpful for the development of the BEV system (Pennock et al., 1984; Smith et al., 1983c). These included (i) the rod-shaped nature of the baculovirus

### Table 1. Milestones leading to the invention of the baculovirus expression system

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<tr>
<th>Description</th>
<th>Impact for research and development</th>
<th>References</th>
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<tbody>
<tr>
<td>Isolation of AcMNPV</td>
<td>Initial discovery</td>
<td>Vail et al. (1971)</td>
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<td>Establishment of first insect cell lines</td>
<td>In vitro studies</td>
<td>Gaw et al. (1959), Grace (1962), Hink (1970)</td>
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<td>Infectivity of haemolymph for cells in culture</td>
<td>BV/ODV difference</td>
<td>Vaughn &amp; Faulkner (1963), Henderson et al. (1974)</td>
</tr>
<tr>
<td>Characteristics of virus produced in cell culture</td>
<td>Infection in cell culture</td>
<td>Brown &amp; Faulkner (1977)</td>
</tr>
<tr>
<td>AcMNPV plaque assay</td>
<td>Purification of genetic variants</td>
<td>Burand et al. (1980)</td>
</tr>
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<td>Infectivity of baculovirus DNA</td>
<td>Transfection</td>
<td></td>
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<tr>
<td>Physical map of AcMNPV</td>
<td>Cloning of individual fragments</td>
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<tr>
<td>Polyhedrin protein is not essential in cell culture</td>
<td>Use of polyhedrin promoter for foreign gene expression</td>
<td>Smith et al. (1983b)</td>
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http://vir.sgmjournals.org
virion that potentially allowed for the insertion of large pieces of extra DNA (in contrast to icosahedral viruses),
(ii) the fact that the polh gene had a very strong promoter
which was active after BV production and hence (iii) that
polyhedrin was not needed for virus amplification in cell
culture. In addition, the absence of the polyhedrin protein
in cells infected with the recombinant viruses could be used
as a visible marker for the selection of the recombinant
viruses (Fig. 1b). The presumed inherent safety of this
insect-virus-based expression system was also mentioned in
1983 (Smith et al., 1983c), based on the knowledge that
baculoviruses are non-pathogenic to vertebrates.

Baculovirus very late gene expression

The TAAG motif was first recognized as a transcription
start site when studying the baculovirus p10 gene (Kuzio
et al., 1984), which had been mapped just previously
(Rohel et al., 1983; Smith et al., 1983c). The P10 protein
was shown to be produced in large amounts, and to form
fibrillar structures in the nucleus and cytoplasm (Fig. 1a),
which were required for efficient OB release from infected
cells (van Oers & Vlak, 1997). The requirements for polh
promoter activity, including a similar TAAG motif, were
analysed in detail a few years later (Ooi et al., 1989).

As indicated above, protein profiles of infected cells sug-
gested three temporal classes of gene expression. Sub-
sequently, detailed analysis of the regulated cascade of gene
expression showed that there were in fact four classes of
genomes: early, delayed early, late and the special class of
very late genes, which comprised the polh and p10 genes (Friesen
& Miller, 1985; Mainprize et al., 1986; Rice & Miller, 1986;
Rohel & Faulkner, 1984). Early genes were expressed in the
presence of DNA synthesis inhibitors (i.e. before replica-
tion), whilst late and very late genes were inhibited by
aphidicolin. Early viral genes contain promoters recognized
by host RNA polymerases. Eighteen baculovirus genes were
shown to be needed for late viral gene expression (Todd
et al., 1995). Both late and very late genes were found to
contain TAAG motifs in their promoters. These genes were
transcribed only by a virus-encoded RNA polymerase
complex, which consisted of four subunits: the late essential
factor (LEF)-4 (capping enzyme), LEF-8 (RNA polymerase),
LEF-9 and P47 (helicase) (Guarino et al., 1998a, b; Passarelli
et al., 1994). The expression of the very late genes also
required very late factor (VLF)-1 (McLachlin & Miller, 1994)
and the host factor polyhedrin promoter binding protein
(PBP) (Ghosh et al., 1998).

The class of very late genes is unique to baculoviruses. The
two very late genes are dispensable for progeny virus
production that takes place in the late phase. This implies
that the very strong polh and p10 promoters can be used in
the context of a baculovirus infection to drive foreign gene
expression (Fig. 2), at their authentic locus or elsewhere on
the viral genome and even in multiple copies.

Early barriers to application and
commercialization

Bacterial and yeast expression systems were up and running
at the time of the development of the BEV system. A
publication on the application of vaccinia virus as an
expression vector appeared in 1982 (Mackett et al., 1982).
These circumstances may have hampered the broad-scale
introduction of yet another expression system, despite its good properties and potential advantages. The commercialization of insect-cell-produced IFN, although IFN was in high demand at the time, was compromised by prior investments in the development of this compound from a bacterial source (Goeddel et al., 1980; Taniguchi et al., 1980). ‘Unknown makes unbeknown’ and without trained people that could work with this new system, it proved difficult to implement this invertebrate system in many laboratories, especially in the early years. Implementation was also hindered because recombinant virus purification required specific skills and commercial kits were not yet available.

The use of the polh promoter for foreign gene expression was patented by the inventors (Smith & Summers, 1984, 1988, 1989). The fact that licensing fees and royalties had to be paid for any commercial use may have slowed down the application of BEV technology, although this was offset to a large extent by the free availability of the system for research purposes. As an alternative, some research groups and companies used the p10 locus and promoter to express foreign genes. When using the p10 locus to insert foreign genes, marker genes such as lacZ were often co-introduced to be able to discriminate recombinant viral plaques (Vlak et al., 1990). The p10 promoter was, for instance, used to produce the classical swine fever virus (CSFV) E2 protein (at the time still called E1) (Hulst et al., 1993), which was later marketed as a subunit vaccine by Bayer (Table 3). The classical swine fever marker vaccine currently on the

![Fig. 2. Typical protein gel electrophoresis result with AcMNPV expression vectors. The protein content is shown for mock- and WT-infected insect cells in comparison with cells infected with expression vectors based on the p10 or polh promoter. The polyhedrin and P10 proteins are indicated as well as a recombinant protein product (β-galactosidase in this case).](http://vir.sgmjournals.org)

<table>
<thead>
<tr>
<th>Property</th>
<th>Transgenic insect cells</th>
<th>Baculovirus vectors in insect cells</th>
<th>Mammalian cells (transient)</th>
<th>Vaccinia vectors</th>
<th>Lentivirus vectors</th>
<th>Bacterial cells (E. coli)</th>
<th>Yeast (Pichia pastoris)</th>
<th>Downstream processing efforts</th>
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<tbody>
<tr>
<td>Post-translational modifications*</td>
<td>++</td>
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<td>+++</td>
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<td>+++</td>
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<td>Homogeneity of N-glycans</td>
<td>++</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>-</td>
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<tr>
<td>Biological activity</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<td>Immunogenicity</td>
<td>++</td>
<td>++</td>
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<td>Production levels</td>
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<td>++</td>
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<tr>
<td>Safety concerns</td>
<td>++</td>
<td>++</td>
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<td>+</td>
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<tr>
<td>Downstream processing efforts</td>
<td>+</td>
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*Post-translational modifications resembling those in mammalian cells.
†Expression levels per culture volume vary per product produced.
‡Removal of viral vectors requires extra processing.
Table 3. Overview of approved vaccines and therapies based on baculovirus expression technology

<table>
<thead>
<tr>
<th>Product name</th>
<th>Company</th>
<th>Expressed product</th>
<th>Purpose</th>
<th>Use</th>
<th>Year of release</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcilis Pesti</td>
<td>MSD Animal Health</td>
<td>E2 glycoprotein</td>
<td>Subunit/marker vaccine against classical</td>
<td>Pigs</td>
<td>1998</td>
<td>–</td>
</tr>
<tr>
<td>Bayovac CSF E2*</td>
<td>Bayer Biologicals/Pfizer Animal Health</td>
<td>E2 glycoprotein</td>
<td>Subunit/marker vaccine against classical</td>
<td>Pigs</td>
<td>2001</td>
<td>Hulst et al. (1993)</td>
</tr>
<tr>
<td>Circumvent PCV†</td>
<td>MSD Animal Health</td>
<td>Porcine circovirus ORF2</td>
<td>VLP vaccine against porcine circovirus type</td>
<td>Pigs</td>
<td>2005</td>
<td>–</td>
</tr>
<tr>
<td>CircoFLEX</td>
<td>Ingelvac</td>
<td>Porcine circovirus ORF2</td>
<td>VLP vaccine against porcine circovirus type</td>
<td>Pigs</td>
<td>2008</td>
<td>Desrosiers et al. (2009)</td>
</tr>
<tr>
<td>Porcilis PCV†</td>
<td>MSD Animal Health</td>
<td>Porcine circovirus ORF2</td>
<td>VLP vaccine against porcine circovirus type</td>
<td>Pigs</td>
<td>2009</td>
<td>–</td>
</tr>
<tr>
<td>Provenge (sipuleucel-T)</td>
<td>Dendreon</td>
<td>PAP-GM-CSF†</td>
<td>Immunotherapy against prostate cancer</td>
<td>Men</td>
<td>2010</td>
<td>Kantoff et al. (2010)</td>
</tr>
<tr>
<td>Glybera</td>
<td>UniQure</td>
<td>AAV vector with lipoprotein lipase transgene</td>
<td>Gene therapy against familial lipoprotein lipase deficiency</td>
<td>Humans</td>
<td>2012</td>
<td>Haddley (2013)</td>
</tr>
<tr>
<td>Flublok</td>
<td>Protein Sciences</td>
<td>Influenza HA</td>
<td>Annual trivalent flu vaccine</td>
<td>Humans</td>
<td>2013</td>
<td>Cox (2009), Cox &amp; Hashimoto (2011), Treanor et al. (2011)</td>
</tr>
</tbody>
</table>

*Bayovac CSF E2 vaccine has been discontinued.
†The two porcine circovirus vaccines produced by MSD Animal Health are licensed in different geographical areas.
‡Prostatic acid phosphatase coupled to granulocyte–macrophage colony-stimulating factor.
market is produced by MSD Animal Health (Porcilis Pesti) and is also based on \( p10 \) promoter-driven expression of the CSFV E2 sequence at the \( p10 \) locus. The \( polh \) and \( p10 \) promoter can also be used simultaneously for foreign gene expression, which was first demonstrated by co-expressing influenza neuraminidase (\( polh \) promoter) and haemagglutinin (HA) (from an extra \( p10 \) promoter copy at the \( polh \) locus) (Weyer & Possee, 1991).

The original patent on the BEV system has since expired, facilitating commercial application of this expression system by many more companies. Diagnostic applications of recombinant proteins produced with the BEV system should be less restricted now, unless the protein itself has been patented. The principle of subunit (e.g. Flublok)- and virus-like particle (VLP)-based vaccines (e.g. Cervarix) for human use will become better known to both professionals and the target public over time, making public acceptance of future recombinant vaccines using baculovirus expression technology probably less difficult.

**Development of baculovirus vectors**

In the early years, a complication of baculovirus technology was the low level of recombinant virus generated in the co-transfection approach (typically 0.1–1%). Therefore, time-consuming purification of recombinant virus was needed, requiring repeated rounds of plaque assays and with the intrinsic danger of losing the foreign gene. A major improvement came when a unique restriction site was inserted at the \( polh \) locus, allowing linearization of the genome (Kitts et al., 1990). A similar approach was also developed for the \( p10 \) locus (Martens et al., 1995). This advance reduced the infectivity of the parental viral DNA. Circular recombinant viral genomes with restored infectivity were generated upon recombination with a co-transfected transfer vector. The percentage of recombinant viruses increased to \( \geq 30\% \) when using this method.

Through further engineering, a vector was constructed in which an essential gene located downstream of the \( polh \) locus (\( orf1629 \)) was disrupted during linearization of the viral DNA by restriction enzyme digestion. The genome was only recircularized upon recombination with an appropriate transfer vector, thereby restoring the \( orf1629 \) gene, leading to an infectious progeny virus. As a consequence, this strategy dramatically reduced the background of WT virus after co-transfection to \( \sim 2–3\% \) (Kitts & Possee, 1993).

In 1993, the ‘bacmid system’ was developed, which allowed the generation of recombinant AcMNPV genomes in \( E. coli \) (Luckow et al., 1993). This method made use of site-specific recombination at the \( polh \) locus by using a bacterial artificial chromosome (BAC) carrying the entire AcMNPV
genome sequence. This development greatly simplified the procedure of making recombinant baculoviruses. As the generation and purification of recombinant viral genomes was performed in bacterial cells, the up-and-ready recombinant genome (single genotype) could be used to transfect insect cells in order to generate recombinant BVs. Bacmids are now used widely, not only to generate expression vectors, but also to generate viruses with gene knockouts for functional studies or to delete undesired ORFs that may reduce the quality of the recombinant protein (e.g. Ono et al., 2012). Bacmid systems have also been developed for an increasing number of other baculovirus species, including BmNPV (Motohashi et al., 2005) and Spodoptera exigua multiple nucleopolyhedrovirus (Pijlman et al., 2002). Major advantages of the bacmid system are that the virus produced is genetically well-defined and that the bacmid can be mutated/ adapted using methods developed for bacterial systems. The use of the bacmid system for the production of proteins for human applications may be limited to preclinical research due to the presence of bacterial sequences and antibiotic selection markers in the viral DNA, and the relative instability of bacmid-derived AacNPV vectors in insect cells (Pijlman et al., 2003a, see below). Therefore, BEVs used in commercial production are still being made by classical recombination with linearized vectors followed by several rounds of plaque purification. A system that combines the possibility of baculovirus genome modification provided by a bacmid set-up with homologous recombination in insect cells, thereby avoiding the bacterial sequences in the final vector, is flashBAC (Hitchman et al., 2009). This method also allows automated gene expression.

The BEV system has seen a new development in recent years with the MultiBac system, which allows the synthesis of multisubunit protein complexes using a single baculovirus vector (Berger et al., 2013). MultiBac technology can be combined with the OmniBac transfer plasmid that can be used universally to generate recombinant baculoviruses, either by homologous recombination using linearized genomes or by applying bacmid technology (Thimiri Govinda Raj et al., 2014). Alternatives are multilocus baculovirus vectors, in which large foreign genes can be inserted at several loci within the baculovirus genome (Galibert et al., 2012; Kanai et al., 2013). These systems have great potential for the manufacture of complex VLPs and the development of multivalent vaccines.

Adaptations for improved glycoprotein expression

Insect cells are well suited for the expression of glycoproteins, and biologically active and immunogenic proteins are often produced. Nevertheless, the levels of production are normally lower for membrane-bound and secreted proteins than for cytoplasmic recombinant proteins. In this respect, it is important to realize that the baculovirus vector encodes the protease pro-v-cath, which accumulates in the ER and may, upon activation by chitinase, interfere with the integrity of recombinant glycoproteins (Hom et al., 2002). When the corresponding v-cath gene is deleted from the baculovirus genome, glycoproteins are less prone to degradation. Moreover, simultaneous deletion of the neighbouring chi-A gene, encoding chitinase, enables higher production levels of recombinant glycoproteins (Hitchman et al., 2010; Kaba et al., 2004). A likely explanation for the latter observation is that massive accumulation of chitinase in the ER may compromise the glycoprotein export machinery (Thomas et al., 1998). Modified baculovirus vectors from which chi-A or both chi-A and v-cath genes have been deleted are available, and are recommended for recombinant glycoprotein synthesis.

Glycoproteins produced in insects generally display more uniform, but less complex N-glycans than mammals (for extended reviews, see Harrison & Jarvis, 2006, 2007). Insect N-glycans have terminal mannose residues, whilst mammalian N-glycans often have terminal sialic acid residues and often show more antennal diversity (Fig. 3). These differences in N-glycan processing may affect the biological function of particular recombinant proteins of mammalian origin produced in insect cells. Transformed insect cell lines have been developed that express a combination of human N-glycan processing enzymes to ‘humanize’ glycoprotein processing in insect cells (Breitbach & Jarvis, 2001; Hollister et al., 1998; Jarvis & Finn, 1996; Mabashi-Asazuma et al., 2013; Okada et al., 2010). Another approach is SweetBac, in which glycoprotein processing enzymes are co-expressed with the gene of interest from the same baculovirus backbone using the above-mentioned MultiBac vectors (Palmberger et al., 2012). Insect cell lines may add core 1,3-α-fucose during N-glycan processing (Long et al., 2006; Tomiya et al., 2004). In nature, 1,3-α-fucose forms the main allergenic component in several insect venoms (Fig 3c) and plant allergens (Altmann, 2007). Two recent papers have addressed this issue by developing baculovirus vectors that express a bacterial enzyme that reduces the amount of the fucose precursor (Mabashi-Asazuma et al., 2014; Palmberger et al., 2014).

Initial attempts have also been made to enhance the levels of functional glycoprotein production by co-expressing human chaperones that can assist in folding. Whilst this may not be needed for the majority of the proteins, it may be helpful for proteins with complex structures, such as those with multiple transmembrane domains (e.g. Tate et al., 1999). However, the levels of improvement achieved so far are limited and the approach was only tested for a few proteins. In-depth knowledge on protein folding in insect cells as compared with mammalian cells is required to enable further advances.

Insect cells infected with baculoviruses that encode human BCL2 (Ahemri et al., 1992) or polydnavirus ankyrin (Fath-Goodin et al., 2006) – both proteins with anti-apoptotic properties – remain viable longer after infection. As a consequence, co-expressing these factors with a gene of interest may enhance the final level of the product. A similar effect on extended cell viability and an increased yield of secreted recombinant protein was observed when co-expressing the insect translation initiation factor eIF4E (Teng et al., 2013).
This mRNA cap-binding protein is known as the rate-limiting factor in translation initiation and is downregulated during infection (van Oers et al., 2001). Overexpression of eIF4E may indirectly promote expression of a broad range of cellular proteins that assist in protein production and cell maintenance.

**Biotechnological aspects**

Scale-up of the infected cell culture is required in order to produce sufficient quantities of recombinant proteins. For small- to medium-scale production, adherent insect cells can be cultivated in large T-flasks, cell stacks or roller bottles. However, suspension cultures are preferred when it comes to large culture volumes. There are numerous systems to grow insect cells in suspension, ranging from shaker flasks and single-use wave bags to stirred tank reactors, which are usually stainless steel fermenters of up to a few thousands litres. Insect cells are shear sensitive (Tramper et al., 1986), but this problem has been largely solved by using appropriate media. Single-use disposable technology is used more and more, and is particularly useful for medium-scale production or as an intermediate step before large-scale production. Disposable technology has the advantage of a shorter downtime between runs because cleaning/sterilization of the fermenter is no longer required (Shulda & Gottschalk, 2013). Baculovirus expression technology does not require equipment that is vastly different from that already used in the pharmaceutical industry. Interestingly, it has recently been shown that redundant or obsolete infrastructure in the pharmaceutical industry can be modified in a short time frame (<3 months) to serve emergency vaccine production with the BEV system (B. Buckland and others, unpublished results).

For suspension cultures, the insect cells are cultivated in serum-free media specifically designed for non-attached cell growth (Weiss et al., 1995). Lepidopteran cell lines derived from the fall army worm (Spodoptera frugiperda; Sf21 and its clone Sf9) and the cabbage looper (Trichoplusia ni; High Five (Hi5)) are used commercially. Most companies create their own proprietary media and use a selected expression clone of a cell line suitable for their particular product. For example, a Sf9-derived cell clone designated expresSF+ is used for the production of Flublok (Protein Sciences) and Glybera ( UniQure). GlaxoSmithKline produces its cervical cancer vaccine in the T. ni Hi5-derived Rix4446 cell line and MSD Animal Health uses Sf-21-CB cells to produce their veterinary vaccine Porcilis Pesti. Whilst many other lepidopteran cell lines have been generated over the past 30–40 years (van Oers & Lynn, 2010), most are only suited for baculoviruses other than the type species AcMNPV. The novel cell line BTI-Tnao38 derived from T. ni (Hashimoto et al., 2012) does support efficient AcMNPV replication associated with high protein production levels and these cells may find further applications in the future.

Large-scale commercial production with baculovirus vectors is carried out in batch mode and is preceded by a number of steps to scale-up the required number of cells. An important factor that determines the efficiency of infection is the cell density (Bernal et al., 2009). In recent years, production at high cell densities, resulting in higher final yields per volume, has been demonstrated successfully by carefully controlling metabolic fluxes (Carinhas et al., 2010). In general, the batch mode production method is reproducible, uses manageable volumes of seed virus and has a production end point that is well defined. Fed-batch systems are not often used for baculovirus expression, but they may support higher cell densities and higher yields (Meghrous et al., 2009). Continuous insect cell bioreactors can be operated to produce recombinant proteins, using a set-up in which a cell growth reactor feeds fresh cells into the baculovirus infection reactor (van Lier et al., 1990). However, this system has not been adopted by the industry, mainly due to the lytic nature of the baculovirus infection and the reproducible, dramatic drop in production that is associated with extensive passaging of the recombinant baculovirus.

It has been well documented that a high m.o.i. or prolonged passage of baculovirus promotes the accumulation of defective interfering (DI) particles (Kool et al., 1991; Lee & Krell, 1992). DI particles replicate at the expense of the intact baculovirus, and cause a reduction in the amount of infectious virus and protein production losses – a phenomenon that is also known as the ‘passage effect’. Infection at low m.o.i. and the use of low-passage seed stocks may largely prevent this phenomenon, but DI particles are rapidly generated within a single virus passage and their negative effects sometimes appear in as little as five to 10 passages (Pijlman et al., 2001). Recombinant baculovirus genomes may also become unstable, resulting in the selective deletion of the transgene upon replication in insect cells. The classical way of generating recombinant baculoviruses via homologous recombination in insect cells is sometimes associated with transgene loss, but the instability problem is most apparent with the highly successful bacmid technology. In recombinant bacmids, the transgene is cloned adjacent to a highly unstable mini-F replicon element and loss of production can happen very fast (Pijlman et al., 2003a). Several technological solutions have been shown to delay DI particle production in small-scale laboratory settings (Pijlman et al., 2002, 2004, 2006), but so far none of these have been translated into industrial practice.

**Quality control, safety aspects and legislation**

Before any product can go to the market and into clinical programmes, many (pre-)clinical tests need to be performed, followed by in-depth evaluation by the responsible authorities, such as the European Medicines Agency and US Food and Drug Administration. The quality control methods and safety aspects related to clinical usage of materials produced in insect cells with baculovirus vectors have recently been reviewed (Roldão et al., 2011). As outlined by Roldão et al. (2011), advanced quality control

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methods are required for the application of these materials as vaccines and therapeutics in order to guarantee safety, stability and constant efficacy over batches. An important consideration for vaccines and therapeutics for human medical use is that the co-produced BV particles need to be cleared away during downstream processing, e.g. by taking advantage of differences in charge between baculoviruses and VLPs or AAV vectors. As an alternative method to overcome the problem of heavy BV contamination, proof of principle has been shown for a BAC-FREE system, in which a gene essential for baculovirus virion (BV and ODV) formation is deleted from the viral genome (Marek et al., 2011). This gene, vp80, is then offered by a trans-complementing cell line to produce a seed stock. Recombinant protein is produced when this seed is then used to infect normal Sf9/Hi5 cells, whilst no baculovirus particles are formed. The vp80 trans-complementation system needs further development, followed by its evaluation for clinically interesting products such as VLPs and AAV vectors; however, if optimized, costs for downstream processing could be reduced considerably.

Specific concerns relate to the possible presence of (retro-) transposons and retrovirus sequences (Menzel & Rohrmann, 2008), and other, persistent, insect viruses as exemplified by the discovery of alphanodaviruses in T. ni Hi5 and Helicoverpa zea Hz-AM1 cells (Bai et al., 2011), and the recent discovery of a novel, insect-specific rhabdovirus in S9 cells (Ma et al., 2014). The presence of insect-specific viruses in established insect cell lines appears to be quite common, and becomes increasingly clear with more sensitive diagnostics and the wide application of next-generation sequencing technologies. For example, Drosophila S2-GMR cells have been shown to be persistently infected with five RNA viruses belonging to four different virus families (Wu et al., 2010). Although these insect-specific, persistent viruses cannot replicate in vertebrate cells, and thus are unlikely to be harmful to humans and other vertebrates, they should be removed with conventional purification systems. As a consequence, there is a demand for new, virus-free insect cell lines.

Rules for the use of baculoviruses to deliver genes into humans for gene therapy or as agents for cancer treatment (see below) have not yet been formulated. Current regulations for proteins produced with BEVs may apply for gene therapy vectors as well, but there are additional safety aspects that need to be evaluated. These include risks of sequence integration, transposon transfer and immunological responses to baculovirus particles (reviewed by Lesch et al., 2011a). To circumvent the activation of the complement system, chemical compounds or soluble complement receptors can be added during transduction (Hofmann & Strauss, 1998; Hofmann et al., 1999). A further innovation has been the development of ‘stealth’ baculoviruses that avoid the vertebrate complement system by displaying complement-degrading proteins on the virus surface (Kaikkonen et al., 2010). The current production procedures may not always be appropriate to produce baculovirus-based gene therapy vectors. An example is the requirement of filter-sterilization for medical products through filters with 0.2 μm pores—a pore size that interferes with the integrity of baculovirus particles. The international community will need to critically evaluate the existing procedures, and may have to develop and agree on new quality control methods and standards to make this application feasible without compromising on safety.

Comparison of the BEV system with other heterologous expression systems

The performance of various expression systems for producing recombinant proteins for biopharmaceuticals has recently been reviewed (Assenberg et al., 2013). The main conclusion from that review is that all three expression systems (E. coli systems, baculovirus technology, and transient and stable expression in mammalian cells) have seen major improvements over recent years allowing higher success rates, increased protein yields and better quality, over shorter time lines. A recent overview of various insect cell expression technologies, including Drosophila S2 cells, is also available (Kollewe & Vilcinskas, 2013). The system of choice of course depends on the characteristics and requirements of the desired product, but will also be influenced by the in-house experience of the researchers. Table 2 shows how the various expression systems compare.

Overview of current applications of BEV technology

The applications of recombinant baculoviruses have recently been reviewed (Airenne et al., 2013), and are very diverse and almost limitless. The applications can be divided into four categories: the synthesis of single or multisubunit recombinant proteins, the production of baculovirus particles as antigen carriers, the manufacture of viral vectors for gene therapy and the production of baculovirus-based gene delivery vehicles for mammalian cells (Fig. 4).

Use of BEVs for the synthesis of single or multipartite recombinant proteins

This first category (Fig. 4a) consists of recombinant proteins used for enzymic analysis, crystallography, diagnostics, subunit vaccines and VLPs, biosensors and protein microarrays. The insect-cell-produced vaccines approved for veterinary or human clinical use are summarized in Table 3, and many more are in (pre-)clinical trials. Table 3 also includes details of a protein applied ex vivo to cure prostate cancer (Provenge). The subunit marker vaccine against classical swine fever contains a secreted form of the CSFV glycoprotein E2. The vaccines against porcine circovirus are based on VLPs that are produced upon expression of the porcine circovirus ORF2. The human papillomavirus L1 capsid protein also forms VLPs when expressed in insect cells. VLPs of human papillomavirus serotypes 16 and 18 form the major component of a vaccine against cervical cancer (Cervarix). In the influenza subunit vaccine Flublok,
the HA protein is present in the form of trimers. These HA trimers further assemble into ‘rosettes’ (Holtz et al., 2003). HA rosettes derived from three different influenza viruses are present in this trivalent seasonal flu vaccine, which is updated annually, analogous to the classical influenza vaccine. More complex VLPs consisting of several proteins can also be produced in insect cells, of which the best-known examples are probably bluetongue and rotavirus VLPs (e.g. Pérez de Diego et al., 2011; and reviews by Palomares & Ramírez, 2009; Vicente et al., 2011). A more recent development is the synthesis of enveloped VLP-based vaccine candidates using BEVs. Examples are recently developed prototype vaccines against chikungunya virus and influenza virus (Metz et al., 2013; Smith et al., 2013). Other complex enveloped VLP-based vaccines are under development (Fernandes et al., 2013; Metz & Pijlman, 2011).

As insect cells are derived from poikilothermic organisms, they have clear potential for the expression of genes from other cold-blooded animals such as fish and amphibians or their pathogens, as recently demonstrated. Salmonid alphavirus structural proteins are only correctly processed to form VLPs by reducing the temperature to 12 °C during the protein production phase (Metz et al., 2011; Hikke et al., 2014). Recent data have also shown that correct folding of influenza HA occurs over a much larger temperature range in Sf9 insect cells than in mammalian cells (Li, 2014), which is also a reflection of the poikilothermic nature of the cells.

**Fig. 4.** Overview of the various applications of the baculovirus expression system. (a) Baculovirus BVs are used to produce foreign (glyco-)proteins in insect cells. The recombinant proteins may be synthesized as subunits, which may remain intracellular or in the case of glycoproteins will be transported to the cell surface or even be secreted. Alternatively, the recombinant proteins may form VLPs or enveloped, secreted VLPs (eVLPs). Protein subunits as well as (e)VLPs may be used as vaccines. (b) When proteins are fused to the BV surface protein GP64 as a carrier, they are transported to the cell surface and become incorporated in the BV particles upon budding. This is called baculovirus surface display. (c) Baculovirus vectors can also be used to produce viral vectors such as AAV for gene therapy. Here, the triple baculovirus system is displayed. Two baculovirus vectors induce the expression of AAV rep and cap genes, respectively, needed to amplify and package the transgene DNA construct encoded by the third baculovirus. GOI, gene of interest. (d) BVs carrying a gene of interest under a promoter active in mammalian cells (MC) are produced in insect cells and used as gene delivery vectors. The nucleus of the insect cell contains the occlusion-derived form of the virus (ODV), which is not occluded in the absence of the polyhedrin protein. (The various components are not to scale.)
Baculovirus particles as antigen carriers

The production of baculovirus particles as antigen carriers is achieved by coupling (glyco-)proteins or epitopes thereof to the BV envelope protein GP64 by means of gene fusion (Fig. 4b). Thus, foreign glycopolypeptides/epitopes will be routed efficiently to the cell membrane to be displayed on the surface of infected cells and on BV particles (Boublík et al., 1995; Grabherr et al., 1997). An extra copy of the gP64 ORF cloned downstream of the polh promoter is used for these modifications to obtain high expression levels and at the same time avoid loss of BV infectivity. Alternatively, heterologous sequences may be coupled to other surface proteins such as influenza HA or the vesicular stomatitis virus G protein (reviewed by Grabherr and Ernst, 2010). Recombinant BVs or cell extracts made with this surface display technique have been used in a variety of applications, including functional studies of glycopolypeptides, drug screening and development of vaccine candidates (see, e.g. Kaba et al., 2003; Mäkelä & Oker-Blom, 2008). This strategy may also be used to target baculovirus-based gene delivery vectors (see below) by coupling peptides specific for target cell receptors to viral surface proteins.

Production of heterologous viral vectors in insect cells

The BEV system finds an important application in the production of other viral vectors (Fig. 4c), such as AAV vectors (Galibert & Merten, 2011; Kotin, 2011; Urabe et al., 2002). The baculovirus system provides a scalable alternative to transfection-based mammalian cell systems to produce AAV vectors for gene therapy applications. Originally, three different recombinant baculoviruses were used to infect insect cells: one with the transgene flanked by inverted terminal repeats, one providing the capsid genes to package the vector DNA and one providing the rep genes needed for production of the vector DNA (Urabe et al., 2002). Over time, the number of baculoviruses needed has been reduced, first to two baculoviruses (Smith et al., 2009) and, more recently, one with the Monobac system (Galibert et al., 2012), where different loci in the baculovirus genome are used simultaneously to express foreign genes. The first approved AAV-based gene therapy vector produced in insect cells (Table 3) aims at the treatment of the rare recessive genetic disorder ‘lipoprotein lipase deficiency’ (Glybera) (Haddley, 2013).

Baculovirus-based gene delivery vehicles for mammalian cells

AcMNPV has the ability to enter mammalian cells by a process called transduction (Hofmann et al., 1995; van Loo et al., 2001). By replacing the polyhedrin promoter with a mammalian (viral) promoter, baculovirus vectors can be used to efficiently express genes in mammalian cells (Fig. 4d) (e.g. BacMam vectors; Condrey et al., 1999). The baculoviruses do not replicate in these cells and the genomes appear as episomal DNA, although occasional integration of baculoviral DNA sequences has been reported (Merrihew et al., 2001). These vectors may be further modified to display mammalian receptor-binding peptides on the surface of BVs (as indicated above) or to incorporate complement-inhibiting factors in the BV envelope (Kaikkonen et al., 2010). A further benefit is the possibility to insert large stretches of foreign DNA, in multiple segments, if necessary, into the viral genome. The use of gene delivery vectors finds applications, for instance, in high-throughput screening of gene functions (human glycopolypeptide project) and the testing of drugs, and may in the future be used for treatment of human pathologies, such as prostate cancer (Swift et al., 2013) and genetic eye diseases (Kinnunen et al., 2009), or assist in bone or wound healing (Chuang et al., 2007; Lin et al., 2010) or transplantation therapies (Murguia-Meca et al., 2011). In addition, baculoviruses are being used to deliver lentivirus replicons into mammalian cells (Lesch et al., 2008, 2011a).

Views on future developments

The development of BEV technology over time is a typical example of new technology entering the scene, with a lag time followed by huge interest, and finally stabilization and incorporation in common laboratory and industrial practice. However, as with all platform technologies, maintenance of the system is not enough and new avenues for improvement need to be explored. Apart for several aspects mentioned above, such as the development of a baculovirus virion-free system to simplify downstream processing and aspects dealing with safety and legislation, the avenues for which we see most potential for optimized expression vectors are minimizing the size of the baculovirus genome, reducing genome instability and overcoming protein-specific hurdles.

Minimized baculovirus genomes

Proteomic analysis of BVs versus ODVs has indicated quite a few ODV-specific proteins, such as the per os infectivity factors (Hou et al., 2013); these ODV-specific proteins are, in principle, dispensable for BV production and over-expression of recombinant proteins. Genes involved in OB formation (in addition to polh), such as the pp32 gene encoding the OB calyx protein, are also dispensable for replication and transmission in insect cells. The same holds true for the aforementioned genes required for the release of OBs from the insect body (chi-A, v-cath and p10). In addition, genes involved in the manipulation of insect host development and behaviour, such as egt and cath (Cory et al., 2002; Li & Guarino, 2008; van Houte et al., 2012), can probably be omitted in the context of protein expression in cultured cells. In the case of AcMNPV, an estimated 40 genes could be eliminated without affecting heterologous protein synthesis and levels. Removal of unnecessary viral genes may allow more space for multiple gene insertions, increase production yields by saving energy, and improve product quality and safety. Removal of unnecessary or even deleterious viral genes has been done so far by making sequential gene deletions from the AcMNPV bacmid, e.g. as
described for a vector without chi-A, v-cath and p10 (Galibert et al., 2011), which made use of an antibiotic marker gene that was removed subsequently by a modified Cre–lox recombination strategy (Suzuki et al., 2007) after each gene inactivation. However, sequential removal of viral genes is a technical challenge and may affect the transcriptional regulation of neighbouring genes or result in increased promoter densities, further disrupting transcription regulation. Alternatively, a ‘building block’ principle may be followed, only including those genes needed for baculovirus replication, BV formation, cell-to-cell transmission and very late protein synthesis. Such a method seems feasible as baculoviruses are generally organized as a sequence of gene cassettes providing single unspliced transcripts, although the presence of co-terminal 5′ and 3′ ends complicates matters (Friesen & Miller, 1985). Using synthetic biology methodologies, baculoviruses may be redesigned and produced synthetically to contain only the essential genes for high-level protein synthesis (Vijayachandran et al., 2013). The latter is also a highly challenging approach as timing and levels of expression of the different genes need to be controlled carefully, but the outlook is very inviting. Both approaches, i.e. sequential deletion and synthetic design, are attractive avenues. In minimal genomes, the DNA sequence motifs required to nucleate viral genome packaging need to be incorporated in order to obtain infectious BVs. Therefore, more fundamental knowledge is needed on baculovirus packaging signals and the proteins required for this process, which are only now beginning to be elucidated (Marek et al., 2013).

Reducing genome instability

Genome instability is an intrinsic property of the WT virus as soon as it is passaged in cell culture. A possible reason is that, in the expression system, AcMNPV replicates in a cell line out of its original tissue/organ context (Pijlman et al., 2003b), which prompts the search for cell lines that better respond to the needs of the system. The fact that instability is associated with the loss of large DNA segments from the viral DNA (Kool et al., 1991), as well as with the accumulation of DNA fragments carrying origins of replication (Lee & Krell, 1994), suggests that replication and/or packaging controls are affected. This may have to do with the structure of the viral DNA itself, its replication mechanism and/or signals that are required for orderly packaging of the DNA into virions. As mentioned above, several modifications to expression vectors have been shown to delay DI particle production. In particular, the deletion of the non-homologous region origin of DNA replication seems a promising approach, which has been shown to improve overall genome stability (Pijlman et al., 2002, 2003b). The expression of the gene of interest from the same transcript as the expression of GP64, which is essential for BV formation and infectivity, has been shown to prevent the loss of the heterologous gene (Pijlman et al., 2006). The next step would be to test both approaches in large-scale bioreactors and translate these into industrial practice.

Protein-specific hurdles

A further challenge lies in the expression of the proteins themselves. Although a plethora of recombinant proteins have been expressed successfully, some are poorly made or need advanced solutions, such as the surface display technique (Kaba et al., 2002). Sometimes it is the consequence of protein toxicity, but most of the time it seems to be a protein folding/transport issue. In WT-infected cells, there is massive transport of polyhedrin and p10 to the nucleus of infected insect cells – proteins that are not glycosylated. In most cases, however, recombinant proteins require routing to the ER and Golgi network to be glycosylated. Production levels of glycoproteins are somewhat erratic and certainly not predictable. We know very little about how proteins are transported and folded in insect cells, particularly glycoproteins, and further insight would be very beneficial for proper and authentic production of these proteins. With such knowledge, we may be able to tailor the quality of expression by co-expressing specific chaperones of vertebrate origin – something attempted so far mainly on a trial-and-error basis.

Baculovirus publications following the development of the BEV system

The impact of the BEV system during its 30 year existence is clearly visible from bibliographical data (Fig. 5). A gradual increase is seen in the number of scientific papers dealing with nucleopolyhedrovirus-related research until the mid-1980s. After the invention of BEV technology in 1983, a steady increase in nucleopolyhedrovirus-related literature can be observed, followed by a peak in the late 1990s, with a maximum of 794 papers published in 1997. After 1997, a gradual decrease in the number of papers published per year can be seen, but it is still averaging ~500 papers in recent years. A total of 14 867 papers published between 1950 and the end of 2013 were retrieved from the Scopus database (see legend of Fig. 5 for search criteria). In the bibliographical analysis it is difficult to separate fundamental studies on nucleopolyhedroviruses from those that apply baculoviruses to express foreign genes (Fig. 5, red curve) and it may also not be appropriate to do so. Baculovirus expression technology would not have existed without preceding fundamental research and would not have reached its current status as an easy-to-use general protein production platform without concurrently obtaining in-depth knowledge on the vector and insect cells. However, to gain an idea how many papers deal with baculovirus expression technology in particular, the search term 'baculovirus expression' was used to create the blue curve in Fig. 5. The search returned a total of 8544 scientific papers with a similar profile as the red curve. This graph probably underestimates the use of the expression system, because many scientists use this system in industrial organizations and they may not be encouraged or allowed to publish their data in the scientific literature. A search amongst patents returned >2300 patent applications, claiming recombinant expression using baculovirus vectors, of which 1500 also included the use of insect
cells. For more insight into patents dealing with baculovirus expression technology, see reviews by Hitchman et al. (2009) and Lin et al. (2011).

Conclusions

Thirty years after its inception, the baculovirus–insect cell expression system and its associated technologies form a mainstream platform for the production of recombinant proteins for fundamental and applied science. The thousands of proteins that have been produced successfully with this system provide convincing support that the BEV system is very useful for academia and industry alike, and this is underscored by the commercialization of veterinary and human vaccines. Further reasons for the success of this technology have been the attitude of the baculovirus community to make new insights and innovative materials readily available to whoever is interested, and the inherent safety of the technology for researchers, producers and consumers alike. In conclusion, there is a lot to celebrate on the occasion of the thirtieth anniversary of the baculovirus expression system. However, this review should also be seen as encouragement to take this system further into the next decades by overcoming the remaining challenges, e.g. to optimize BEV genome composition, to improve genome stability in order to guarantee product quality over batches and to simplify downstream processing without losing safety. This will allow the baculovirus expression system to become the system of choice for many applications based on its convenience, and on the yield, quality and safety of the products produced.

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Thirty years of baculovirus expression


