Recent Developments in the Use of Baculovirus Expression Vectors

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Abstract

Over 35 years since it was established to make recombinant proteins, the baculovirus expression vector system continues to develop and improve. Early systems for recombinant virus selection were laborious, but better methods were rapidly devised that enabled non-virologists to use baculovirus vectors successfully in a wide range of applications. These applications include multiple gene expression for complex molecules, production of adeno-associated virus-like particles for gene therapy, the use of baculovirus budded virus for the same purpose, numerous potential human and animal vaccines, and for other therapeutic proteins. A number of products for human and veterinary use are now on the market, which attests to the utility of the systems. Despite these successes, baculovirus vectors essentially remain in a relatively primitive state of development. Many proteins, particularly membrane-bound or secreted products, continue to be difficult to produce. Various research groups are working to identify potential areas of improvement, which if combined into an ideal vector might offer considerable advances to the system. This chapter will review some of the most recent reports and highlight those that might have generic application for recombinant protein synthesis in insect cells. We also summarize parallel developments in host cells used for baculovirus expression and how culture conditions can influence protein production.

Introduction

It is more than 30 years since the first papers were published that described making recombinant proteins in insect cells using modified baculovirus genomes (Smith *et al.*, 1983; Pennock *et al.*, 1984). Since those early days, much progress has been made in making the baculovirus expression vector system (BEVS) more amenable for use in a wide variety of academic and industrial laboratories (van Oers *et al.*, 2015). This undoubtedly has contributed to the widespread use of the BEVS, which is now one of the most popular eukaryotic expression systems. However, the most commonly used commercially available vector platforms, Bac-to-Bac™ (Luckow *et al.*, 1993), in which foreign genes are inserted by targeted transposition from an intermediary transfer plasmid, has been unchanged since its launch as a commercial kit by Thermofisher. However, Mehalko and Esposito (2016) have produced Bac-2-the-Future, a modified version of the system. This version is claimed to improve the efficiency of recombinant virus production by reducing the number of false positives generated in the transposition process. For example, a site for Tn7 transposition was removed from the bacterial host cell used. It has yet to be developed into a commercial kit but clearly has potential. The second most popular BEVS, in which foreign genes are inserted into the virus genome by homologous recombination with a transfer plasmid, has evolved over time. It has spawned a number of commercially available

kits such as *flash*BAC™ (Possee *et al.*, 2008; van Oers *et al.*, 2015), but the core technology of homologous recombination has not altered.

That does not mean, however, that there have been no improvements to the BEVS system in recent years. Rather it indicates that the considerable developments made have largely come from modifications to the intermediary transfer plasmids, to the cell lines used to propagate virus or produce recombinant proteins, or by the incorporation of new technology such as RNA interference (RNAi). Two exceptions are firstly, the development of the MultiBac system used to produce multiple proteins within a single recombinant virus (Berger *et al.*, 2004). Secondly, developments exemplified by the variants of the *flash*BAC system that have led to improvements in the yield and/or quality of particular types of proteins through deletion of non-essential baculovirus genes or have reduced the effort required to make recombinant viruses by removing the need to separate non-recombinant from recombinant viruses (Possee *et al.*, 2008; Hitchman *et al.*, 2010).

This chapter, therefore, focuses on recent improvements that have been made to the BEVS to either overcome some of the challenges posed by particularly difficult-to-express proteins or multi-protein complexes such as virus-like particles (VLPs). Novel uses of the expression vectors beyond the confines of insect cells, including enhancing gene delivery into mammalian cells, gene therapy and applications in vaccine production, are also considered. Table 10.1 summarizes what we consider to be the most significant improvements applicable across a wide range of applications of the baculovirus expression system. Those not included in the table have more specific uses.

Vector development

Multiple expression vectors and virus-like particle production

Baculovirus expression vectors have often been used to produce more than one recombinant protein, which can then interact to form highly complex

Table 10.1 Significant recent advances in baculovirus expression technology

Category	Name	Company	Description	Reference
Virus vectors	biGBac	N/A[1]	Gibson assembly and MultiBac to insert 25 genes into the baculovirus genome	Weissman *et al*. (2016); Weissman and Peters (2018)
	Top-Bac®	Alegenex	Overexpression of AcMNPV *ie1*, combined with tandem late/very late baculovirus gene promoters enhances recombinant protein production	Gómez-Sebastián *et al*. (2014); López-Vidal *et al*. (2015)
	Vankyrin	ParaTechs	Incorporating anti-apoptosis vankyrin genes into the baculovirus vector improves virus-infected cell viability 48–72 hours post infection	Steele *et al*., (2017)
	Synthetic AcMNPV	N/A	Infectious virus genome assembled synthetically	Shang *et al*. (2017)
	Bac-2-the-Future	N/A	Updated version of Bac-to-Bac with improved efficiency of recombinant virus production	Mehalko, J.L. and Esposito, D. (2016)
Cell lines	Nodavirus-free	N/A	*Trichoplusia ni* cell line free of nodavirus	Chen *et al*. (2013)
	Rhabdovirus-free	N/A	*Spodoptera frugiperda* cell line free of rhabdovirus	Maghodia *et al*. (2016)
	Vankyrin	ParaTechs	Incorporating anti-apoptosis vankyrin genes into insect cell line improves protein yields	Fath-Goodin *et al*. (2006); Steele *et al*., (2017)
	One Bac	N/A	Stably transformed Sf9 cells to produce AAV Rep and Cap proteins	Mietzsch *et al*. (2014, 2015, 2017)

[1]Not applicable.

structures such as virus-like particles (VLPs). One of the early examples of this application of the technology was the assembly of bluetongue VLPs (Roy, 1992). More recent studies have made further developments in multiple gene expression. Weissman *et al*. (2016) describe a method for assembling recombinant baculoviruses that could contain up to 25 individual genes of interest within 6 days. The method is based on the Gibson assembly reaction. The method is termed biGBac; biG referring to both the size of the achievable expression construct and the reverse of Gib from Gibson. Bac is a reference to the generation of the baculovirus vector, which is derived from the MultiBac system (Berger *et al*., 2004). This method was used to generate and characterize recombinant forms of the anaphase-promoting complex/cyclosome, cohesin and kinetochore complexes. It should be noted that the genes of interest need to be cloned into an initial plasmid before being usable in the assembly reaction, which can be undertaken using the Gibson assembly reactions or conventional techniques. A detailed, recent review of this method is provided by Weissmann and Peters (2018).

By coincidence, Zhang *et al*. (2016) also targeted expression of the multi-subunit anaphase-promoting complex/cyclosome complex. They utilized a Uracil-Specific Excision Reagent ligation-free cloning method to assemble recombinant MultiBac transfer vectors. Ultimately, 13 proteins were produced by coinfection of insect cells with two recombinant viruses with genes inserted at the polyhedrin gene (*polh*) or chitinase/cathepsin gene loci.

The assembly of the transfer vectors required for such complex expression projects is not a simple task. The approach builds on the MultiBac system (Berger *et al*., 2004). Increasingly, the baculovirus expression system is being used to produce multi-subunit structures that require very careful design and production of gene constructs using synthetic biology.

Multiple expression vectors have also been developed with the goal of improving expression of individual recombinant proteins, which remain the most commonly used application of BEVS. Gómez-Sebastián *et al*. (2014) described a rearranged baculovirus expression vector cassette in which they over-produced the AcMNPV IE1/IE0 transcriptional transactivator using either the *polh* promoter or $pB2_9$, the latter comprising a lower strength promoter from *Trichoplusia ni* insects. In this vector, recombinant gene expression was driven by one of a number of promoters including some hybrid sequences formed from late and very late *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) genes. These modifications resulted in both enhanced recombinant protein production and an increase in virus-infected cell viability and integrity. While this initial study was performed using green fluorescent protein (GFP) as a reporter, a subsequent study showed that production of porcine circovirus type 2 and rabbit haemorrhagic disease calicivirus VLPs could also be enhanced using this system. A yield increase of 300% was quoted for each target (López-Vidal *et al*., 2015). The system is known commercially as Top-Bac®.

Steele *et al*. (2017) described the incorporation of anti-apoptotic genes (vankyrin) into the baculovirus vector to extend the period of recombinant protein production in baculovirus-infected cells. Co-expression of vankyrin with a recombinant protein improved the viability of virus-infected cells at the critical 48–72 hours post-infection (hpi) period when most cultures are harvested. Importantly, the yield of recombinant protein was also higher when using these vectors. This approach is linked to the establishment of insect cell lines that have vankyrin genes inserted into their chromosomes for constitutive production (see below).

Vectors for recombinant adeno-associated virus production

Adeno-associated virus (AAV) is non-enveloped with a single-stranded DNA genome and is classified in the *Parvoviridae* (Hoggan *et al*., 1966). AAV is unable to replicate autonomously and requires another virus helper, which is usually adenovirus or herpes simplex virus (HSV), both of which have large double-stranded DNA genomes. This dependence on another virus further classifies AAV as a member of genus *Dependovirus*. The AAV genome is 4.7 kb and contains two main open reading frames, *rep* and *cap*. The former encodes four regulatory proteins (Rep78, Rep68, Rep52, and Rep40) that play important roles in both replication and the encapsidation of viral DNA. The *cap* encodes three capsid proteins (VP1, VP2, and VP3) and assembly-activating protein (AAP) that promotes the formation of capsids. Each end of

the virus genome is flanked by inverted terminal repeats (ITRs). These contain Rep recognition sequences important for AAV DNA replication and also for the packaging of the genome (Samulski and Muzycska, 2014). The latent state of AAV, where low numbers of genomes are integrated into the host chromosome, has led to the use of recombinant (r)AAV as a practical gene therapy vector and much effort has been made to optimize rAAV production (reviewed by Hastie and Samulski, 2015). In a recent review (Aponte-Ubillus *et al*., 2018), it was concluded that use of the baculovirus expression platform to produce rAAV was one of the most promising, generating the highest yield of virus particles on a per cell basis.

In the first use of baculoviruses to produce rAAV vectors, *rep78* and *rep72* were placed under the control of the AcMNPV *ie1* and *polh* promoters, respectively. The *cap* was placed under the control of another copy of the *polh* promoter, with the VP1 translation initiation codon mutated to ACG to enable synthesis of VP2 and VP3. Finally, the transgene was linked with a gene promoter active in human cells, flanked by ITRs and inserted into a third baculovirus (Urabe *et al*., 2002). Although production of rAAV vectors was successful with this approach, the use of three separate recombinant baculoviruses was undesirable. The requirement for coinfection of insect cells with each virus to achieve AAV formation was impractical for long term applications. Further developments of the baculovirus-AAV production system have seen the number of vectors required reduced to two. The Rep and Cap components have been incorporated into a single virus under the control of *polh* and *p10* promoters, with the transgene in the second virus flanked by ITRs (Chen, 2008; Smith *et al*., 2009).

Wu *et al*. (2018) have reported the use of ribosome leaky-scanning to express AAV Rep and Cap proteins downstream of the *polh* and *p10* promoters, respectively, with the rAAV genome inserted between the two baculovirus promoters in a single recombinant virus. The yields of recombinant serotypes rAAV2, rAAV8, and rAAV9 derived from the single recombinant baculovirus-infected Sf9 cells exceeded 10^5 vector genomes (vg) per cell and the authors suggest that this system would be suitable for large-scale rAAV production. The single virus vector was shown to be stable and showed no apparent decrease of rAAV yield after at least four serial passages (p4). Although a decrease in yield was observed after the fourth passage, p4 is a workable limit for scale-up studies.

When using any rAAV production system, it is important that capsid stoichiometry is as close to wild-type as possible; imbalance results in reduced potency. Bosma *et al.* (2018) generated a library of recombinants with variations in the translation-initiation region of VP1, which resulted in the production of a potent rAAV serotype 5 with a balanced VP1/VP2/VP3 stoichiometry. They also demonstrated that excessive incorporation of VP3 had the highest decrease in potency. The authors suggest that modifications for other serotypes of VP1 could result in the development of high potency rAAV with capsid stoichiometry similar to wild-type virus.

An alternative rAAV production platform in insect cells, referred to as the OneBac system, uses a stably transformed Sf9 cell line to express *rep* and *cap* for specific serotypes. Infection with a single baculovirus containing the AAV vector backbone is, therefore, sufficient to induce rAAV production (Aslanidi *et a*l., 2009; Mietzsch *et al*., 2014, 2015). More recently, Mietzsch *et al.* (2017) have developed this system further by removing the Rep-binding element (RBE) from the *rep/cap* expression constructs, which results in a reduction in the packaging of unwanted foreign DNA into rAAV particles for four serotypes (AAV1, AAV2, AAV5 and AAV8). The RBE was previously believed to be essential for efficient rAAV packaging in Sf9 cells, but the authors' previous studies demonstrated this not to be the case (Mietzsch *et al*., 2015).

Previous work has demonstrated that the use of truncated ITRs has had no impact on rAAV vector production in mammalian cells, hence the baculovirus production system has been based on using truncated ITRs (reviewed by Aponte-Ubillus *et al*., 2018). Recent work by Savy *et al*. (2017) replaced the truncated ITRs with the wild-type version and additional wtAAV2 sequences, which improved the percentage of rAAV8 full capsids from 10% to 40% and demonstrated a 10-fold reduction in non-rAAV encapsidated baculovirus DNA. These factors were considered to mitigate the slight decrease in the overall rAAV titres observed with the constructions containing the wild-type ITRs. The authors also indicated that the position of the ITRs influences

encapsulation, an outcome that was confirmed by the work of Penaud-Budloo *et al.* (2017).

Glybera was the first baculovirus-derived, rAAV-based treatment for a medical condition, a rare familial lipoprotein lipase deficiency (LPLD) (US National Library of Medicines, 2019). A 5-year authorization to market the drug in the European Union was granted in 2012. However, near the expiration of this term, an extension was not sought by the manufacturers owing to its failure to attract customers (Senior, 2017). Perhaps this was not surprising as the cost of the treatment per patient was estimated at around US$1M, although the product was never approved for use in the USA. This example highlights the perils for companies in producing a technically complex drug intended for a very limited market.

A further concern for developers of AAV-based therapies is the potential for contamination of the final product with protein, nucleic acids or other components of the respective host system utilized (Hastie and Samulski, 2015). rAAV particles produced in mammalian systems contained host cell and plasmid DNA (Hüser *et al.*, 2003; Nony *et al.*, 2003). This was a point raised in the European Medicines Agency (EMA) assessment report for Glybera prior to its approval for clinical use. There was concern about the characterization and elimination of baculovirus-derived impurities in the final product. While most studies examining the purity of AAV particles have focused on the use of quantitative polymerase chain reaction and Sanger sequencing, a recent report described the use of high throughput sequencing based on the Illumina platform (Penaud-Budloo *et al.*, 2017). These results showed that 2.1% of the sequence reads were baculovirus in origin, while Sf9 host cell DNA matched less than 0.03% of reads. The authors of this study suggest that their novel method, which they called single strand virus sequencing (SSV-seq), should enable effective DNA quality control for AAV vectors.

The Glybera example also suggests that costs of production need to be reduced considerably. While initial development costs have to be recovered, this has to be done at a rate that is compatible with a reasonable price for the drug. A very important factor in the reduction of costs of producing AAV is the yield of AAV particles. To take into account that some particles may be devoid of DNA, this is usually expressed as vector genomes (vg) per cell. In the original study (Urabe *et al.*, 2002) this was estimated at 5×10^4 vg/cell. In a more recent study, the yield had been increased to 5×10^5 vg/cell (Mietzsch *et al.*, 2014). For comparison, the host baculovirus vector produces about 100 infectious plaque forming units per cell; although total virion numbers may be higher given that some virus particles will be non-infectious.

BacMam vectors

Despite their successful use as expression vectors of foreign genes in insect cells, baculoviruses have also been developed as efficient vehicles for the delivery of transgenes into mammalian cells (Hofmann *et al.*, 1995; Boyce and Butcher, 1996). BacMam vectors utilize mammalian-specific transcription signals such as the human cytomegalovirus (CMV) immediate early gene promoter to drive recombinant protein production. Budded baculovirus particles are able to enter mammalian cells and then uncoat to release virus DNA in nuclei. However, the baculovirus cannot accomplish a complete cycle of replication in mammalian cells to produce progeny infectious virus. Consequently, the BacMam vector system is regarded as an inherently safe means of transducing mammalian, including human, cells. A recent overview of the system has been presented by Mansouri and Berger (2018).

One application of BacMam technology that is showing promise is their potential use as animal vaccines. Conventional inactivated or attenuated vaccines against *African swine fever virus* (AFSV) were unsuccessful or not practical (Neilan *et al.*, 2004). Argilaguet *et al.* (2013) constructed BacMam-sHAPQ, a vector expressing a fusion protein comprising ASFV P54, P30 and secretory haemagglutinin antigens from the E75 virus isolate using the CMV immediate early gene promoter. Promisingly, four out of six immunized pigs showed total protection against sub-lethal ASFV challenge and there was an induced, specific high T-cell response after *in vivo* immunization in surviving pigs.

Zhang *et al.* (2014) constructed a dual expressing BacMam vector (BV-Dual-s1). This produced S1 glycoprotein of avian infectious bronchitis virus (IBV) as a fusion with the AcMNPV GP64 major surface glycoprotein. The hybrid protein was placed under the control of both the CMV early

gene promoter and the baculovirus *polh* promoter. This resulted in the display of the S1-GP64 on the budded virus surface when synthesized in insect cells. However, in avian cells, the S1-GP64 was also expressed owing to the presence of the CMV gene promoter. The BV-Dual-s1 virus also encoded the vesicular stomatitis virus (VSV) G glycoprotein under the control of the AcMNPV *p10* promoter. This resulted in the display of VSV-G on the surface of the budded virus, which was proposed to aid virus uptake into avian cells. Chickens immunized with BV-Dual-s1 produced a significantly higher antibody response when compared to the control groups. Immunized chickens challenged with virulent IBV showed a similar protection rate with BV-Dual-S1 BacMam (83%) when compared to chickens immunized with inactivated vaccine (89%). The BV-Dual-s1 also induced strong humoral and cell-mediated immune response.

Both of the above papers highlight stimulation of the cell mediated immune responses in vaccinated animals, which is often required for complete protection. This was also noted in a study by Heinimäki *et al.* (2017), where monomeric ovalbumin protein and oligomeric norovirus VLPs were inoculated into BALB/c mice with and without infectious budded baculovirus particles. Live baculovirus appeared to act as strong adjuvant for both humoral and cell-mediate responses. The response of the immune system appeared to be less effective if only the proteins or proteins plus inactivated baculovirus were injected. It is suggested that unmodified baculovirus can be an effective adjuvant for a subunit vaccine, which has profound implications for the further development of the system. Ono *et al.* (2018) concluded that the anti-baculoviral responses are induced by recognition of unmethylated CpG virus DNA in mammalian cells. This is mediated by the toll-like receptor (TLR) 9/MyD66/IRF7 in immune cells with the subsequent production of pro-inflammatory cytokines and type 1 interferon.

Vectors to optimise secreted and membrane protein production

The secretion of recombinant proteins by baculovirus expression vectors is often poor, relative to the total amount of protein synthesized in the cell. This is an issue that has long been recognized as a problem (Jarvis *et al.*, 1993). There was an encouraging report from Tessier *et al.* (1991) that secretion of plant propapain from baculovirus-infected insect cells could be improved by utilizing the signal peptide from the honey bee prepromellitin instead of its native sequence. However, this same study also showed that propapain linked with the *Drosophila* α-amylase signal peptide, and β-galactosidase fused with the honey bee prepromellitin signal peptide, failed to be secreted. Jarvis *et al.* (1993) assessed secretion of human tissue plasminogen activator (tPA) utilizing various signal peptides. These included those from the major baculovirus structural glycoprotein (GP64), the insect secretory protein (cecropin B), a luminal protein of the rough endoplasmic reticulum (GRP78/BiP) and the honey bee prepromellitin. None of these improved the secretion of tPA. The lack of secretion of β-galactosidase (Tessier *et al.*, 1991) emphasizes the difficulty of simply adding a signal peptide to another protein and expecting it to be secreted in a heterologous system. However, it is worth recalling that two of the first proteins to be produced using baculovirus vectors, interferon (Smith *et al.*, 1983) and influenza virus haemagglutinin (Possee, 1986) were secreted or plasma membrane-bound proteins that retained their native signal peptides.

Lou *et al.* (2018) approached the problem of secreting human thyroid peroxidase (hTPO) by testing a range of different signal peptides in a manner similar to Jarvis *et al.* (1993). Although hTPO can be produced using baculovirus expression vectors, it largely adopts an aggregated form in the cell lysate fraction despite having its own signal peptide (Fan *et al.*, 1996). Five alternative signal peptides were tested in conjunction with hTPO by Lou *et al.* (2018) using the *T. ni* High Five™ host cell for expression. These signal peptides comprised those from bee melittin, AcMNPV GP64, ecdysteroid UDP-glucosyl transferase (EGT), human peptidyl-glycine alpha-amidating monooxygenase (PAM) or human azurocidin. While EGT and azurocidin signal peptides decreased hTPO secretion, compared with the original native protein, the remaining three served to increase secretion of the protein. The best performer was the PAM signal peptide, which enhanced hTPO secretion about 2.5-fold. This modified form of hTPO in a baculovirus vector was then used in a wave-type bioreactor for scale-up production of active recombinant protein in insect cells, achieving a protein purity of

>95% from the harvested culture medium. While the increase in hTPO secretion may appear modest, it can make all the difference when protein purification is required. What this study also emphasizes is that the selection of a suitable signal peptide sequence is still largely an empirical process. It requires investment in producing several different gene constructs to find the best combination of signal peptide and target protein to achieve optimal secretion.

Chakraborty *et al.* (2018) demonstrated that it is possible to adapt membrane-targeted proteins for secretion by adding signal peptides to the target protein extracellular domain. In this case, they produced secreted versions of *Arabidopsis thaliana* TDR and PRK3 plasma membrane receptors by adding either GP64 or hemolin signal peptide sequences to the extracellular domains. These proteins were subsequently purified and used in X-ray crystallography studies. The authors suggest that such modifications to recombinant proteins could be applied to other membrane proteins in the animal kingdom.

Some studies have attempted to improve production of secretory proteins by utilizing baculovirus promoters that are active earlier in the virus replication cycle, with the expectation that the host cell secretory apparatus is more effective than in the very late stages of infection. Early studies showed promise with this approach, for example, Lawrie *et al.* (1995) demonstrated an increased yield of urokinase using the AcMNPV *p6.9* promoter as did Bonning *et al.* (1994) for secreted juvenile hormone esterase. However, almost all recent studies use the standard *polh* promoter in combination with different approaches to optimize secretion. The *vp39* promoter has also been used in BmNPV expression systems to achieve reduced aggregation of several foreign proteins (Ishiyama and Ikeda, 2010).

Toth *et al.* (2011) conducted an extensive study with *Western equine encephalitis virus* (WEEV) glycoproteins comprising complete E1, E1 ectodomain, E26KE1 polyprotein precursor and an E2E1 chimera. These were placed under control of *ie1*, *p6.9* or *polh* promoters. No glycoprotein expression was observed using the *ie1* promoter and equal amounts of soluble, processed material were produced using the late and very late gene promoters. Thus, earlier expression did not result in large amounts of high quality, soluble recombinant glycoprotein. This study emphasizes the difficulty of making general recommendations about the optimal vector to use for gene expression.

Another approach to improve the secretion of recombinant proteins is to co-express protein chaperones. Ailor and Betenbaugh (1998) partially overcome the problem of insoluble immunoglobulin produced in baculovirus-infected cells by co-expressing cytosolic heat shock protein (hsp) 70 chaperones. The hsp70 was found to co-precipitate with the immunoglobulin, which indicated formation of a specific complex. Solubility of the immunoglobulin in the cell appeared to be increased by the presence of hsp70. Secretion of the immunoglobulin also appeared to be improved. Co-expression of immunoglobulin with BiP (murine chaperone immunoglobulin heavy chain binding protein) also increased the solubility of processed immunoglobulin chains.

More recently, the secretion of a fusion between enhanced GFP and secretory alkaline phosphatase (SEAP) was improved in insect cells by co-expression of the chaperone calreticulin (CALR) or translation initiation factor eIF4E (Teng *et al.*, 2013a). Another report showed that α-synuclein (α-syn) and β-synuclein (β-syn) also enhanced secreted protein production (Teng *et al.*, 2013b). When β-syn + CALR + eIF4E were co-expressed with SEAP, this resulted in a 1.8-fold increase in reporter protein production (Teng *et al.*, 2013c). The authors also noted a prolonged period of protein secretion, up to 10 days pi, using this combination of chaperones and reporter. It would be interesting to test this system with proteins that are poorly secreted when compared with SEAP.

A slightly different approach to studying the potential role of chaperones in the production of recombinant proteins in baculovirus-infected cells was taken by Imai *et al.* (2015). *Bombyx mori* cells were treated with double-stranded RNA specific for BiP, CNX, CRT, ERp57 and PDI to reduce expression of the respective protein chaperone via RNAi. Having demonstrated a reduction in mRNA for these genes in uninfected cells, other cultures were subsequently infected with BmNPV recombinant viruses encoding human erythropoietin (EPO), cat hepatocyte growth factor (HGF) or mouse wingless tumour virus (MMTV) integration site family of member 3A (Wnt3A). Protein secretion was assessed four days pi. Depleting BiP reduced the

secretion of HGF and also decreased the insoluble fraction of EPO. Reducing CRT decreased soluble EPO and increased insoluble EPO. HGF soluble and insoluble forms were decreased by CRT RNAi. The silencing of PDI also reduced the insoluble levels of EPO and HGF.

In further experiments, Imai *et al.* (2015) produced cell lines overexpressing BiP, CNX and ERp57 chaperones. When these cell lines were used as hosts for the three recombinant viruses with their reporter genes the protein expression results when assessed three days pi were mixed. Secreted and soluble forms of EPO were higher in all three of the cell lines overproducing chaperone proteins. Soluble and insoluble forms of HGF were also higher. Soluble Wnt3A was increased if BiP but not CNX or ERp57 were overexpressed. None of the chaperones were able to increase secretion of Wnt3A. The authors concluded that although differences in expression levels were noted, these were not significant.

Fath-Goodin *et al.* (2006) described the benefits of co-expressing anti-apoptotic genes to extend the window of protein production and hence increase yields of proteins, particularly those secreted or membrane-bound. More recently, Steele *et al.* (2017) have described the production of several insect cell lines that incorporate one of these apoptotic genes, vankyrin. The cell lines included SfSWT-4 (Mabashi-Asazuma *et al.*, 2013), which can produce glycoproteins with complex-type N-glycans (see also 'Developments in cell lines to support recombinant protein production'). Vankyrin levels were further increased by also incorporating the *p-vank-1* gene into the baculovirus vector (see also 'Multiple expression vectors and virus-like particle production'). Cell lysis was delayed, and recombinant glycoprotein yield increased when SfSWT-4 cells were infected with a vankyrin-containing expression vector. A synergistic effect resulting in increased glycoprotein levels was observed when the vankyrin gene was expressed both constitutively in the cell line and from the incoming virus vector.

Many secreted and membrane-targeted proteins are glycosylated, and whilst insect cells are capable of producing biologically active and immunogenic proteins, the pattern of glycosylation differs from that in mammalian cells. Glycoproteins synthesized in insect cells produce more uniform and less complex glycans that normally terminate in mannose residues, in comparison to mammalian cell glycans that most frequently terminate with sialic acid (Harrison and Jarvis, 2006). Transformed insect cell lines, such as SfSWT-4 (Mabashi-Asazuma *et al.*, 2013) that have been modified to express mammalian N-glycan processing enzymes have met with some success, whilst an alternative approach has been to encode these enzymes alongside the target glycoprotein using MultiBac vectors (referred to as SweetBac; Palmberger *et al.*, 2012). In the latest developments, Moremen *et al.* (2018) describe an elegant modular approach in which they have created an expression vector library encoding all known human glycosyltransferases, glycoside hydrolases and other glycan-modifying enzymes. The enzymes were expressed as secreted catalytic domain fusion proteins in insect cells (and mammalian cells) and a subset were characterized. This library forms an important future resource for recombinant enzyme production.

Membrane proteins are still regarded as some of the most difficult to express in the baculovirus-insect cell system. Yields tend to be low and subsequent purification somewhat problematic. The efficient production of membrane proteins, particularly those destined for the plasma membrane, shares similar problems to those described above for secreted proteins. Both must enter the secretory pathway of the virus-infected cell and be directed to the plasma membrane. The passage of the plasma membrane bound protein is then halted by a hydrophobic domain normally at the carboxyl end of the sequence. One of the very first proteins made using the baculovirus expression system was the influenza virus haemagglutinin (Possee, 1986). This is moved efficiently to the plasma membrane and is also incorporated into budded virus particles as they leave the cells. Hence, baculoviruses can be used to display plasma membrane proteins (see Chapter 11).

The plasma membrane is not the only destination for membrane proteins. While the final maturation process for influenza and baculovirus virus particles is very similar, requiring the transit of ribonucleoprotein structures or capsids respectively to the plasma membrane and subsequent budding from the cell, others such as HSV are more complex. The molecular basis for the assembly of HSV particles is only beginning to be understood.

Virus capsids leave the nucleus via budding at the inner nuclear membrane to form primary enveloped virions in the perinuclear space. The outer leaflet of the nuclear membrane then fuses with the virion primary envelope to allow their release into the cytoplasm. More than 15 tegument and 10 envelope glycoproteins are added as the virus buds into Golgi-derived vesicles. The mature virions are released when the vesicle membrane fuses with the cell plasma membrane (Mettenleiter, 2006). One of the virus envelope components is the membrane fusion factor glycoprotein B (gB). This is a target for development as an HSV vaccine owing to its role in the fusion of the virus envelope with the host cell during virion uptake (Atanasiu *et al.*, 2010). Human cytomegalovirus (HCMV) is one of the herpesviruses currently targeted for the production of an effective vaccine.

HCMV gB is synthesized as a precursor polypeptide, which is cleaved by furin into the N-terminal surface (SU) and the C-terminal membrane-anchored (TM) domains. These are linked by a disulphide bridge (Heldwein *et al.*, 2006), and anchored to membranes by the hydrophobic helix of the TM chain. The protein is heavily glycosylated. It has 18 potential N-glycosides resulting in about 40% of its mass being comprised of sugars when expressed in human cells (Britt and Vugler, 1989). The structure of gB is a trimer of 360–420 kDa. This complex molecule has been difficult to produce in a recombinant form with particular regard to its use as a vaccine. An HCMV vaccine candidate based on a secreted, truncated form of gB produced in Chinese hamster ovary cells was reported to reduce by 50% the infection risk in a Phase II clinical trial (Pass *et al.*, 2009). This has stimulated further efforts to develop a vaccine.

Patrone *et al.* (2014) were able to produce a full-length form of HCMV gB using the baculovirus-insect cell system. Yields of gB were 4–6-fold higher in High Five™ cells compared with Sf9 cultures. The authors noted that the latter cells were less able to tolerate high densities at the time of virus infection when compared with High Five™ cells. The addition of rapamycin was found to inhibit baculovirus-induced swelling that is commonly observed with this system. The drug also prolonged the intracellular accumulation of gB in the insect cells. Furthermore, the inhibition of cell swelling did not appear to interfere with budded virus assembly and infectivity. Supplementing the culture medium with cysteine further improved the gB yields by increasing the detergent-soluble protein fraction. This was compatible with the enhancements achieved with use of rapamycin. Overall, the use of rapamycin in combination with cysteine supplementation resulted in the production of gB at a milligram scale. The gB was purified in its trimeric post-fusion conformation and is likely a good candidate for use as a vaccine, more closely resembling the native protein. This study also illustrates how even very complex proteins may be expressed and purified using the baculovirus system.

Incorporating RNAi technologies to improve protein production

RNA interference (RNAi) has transformed the way we study gene function (Liu and Paroo, 2010). Although originally intended as a tool to study biological processes (Fire *et al.*, 1998), in recent years it has also been used to improve the yield and quality of recombinant proteins across different expression platforms including CHO (Herbert *et al.*, 2009) and insect cells (Chavez-Pena and Kamen, 2018). In insect virology, instead of making time-consuming deletions to study gene function, RNAi can be used to silence gene expression through targeted destruction of the relevant mRNA. For example, Terenius *et al.* (2011) used this approach to study the different processes involved in virus infection. Baculovirus expression vectors have also been used to deliver dsRNA into mammalian cells (Suzuki *et al.*, 2008).

The limiting factor in the application of RNAi technology in insect cells is the uptake of dsRNA from the culture medium into the cell cytoplasm (Yu *et al.*, 2013). Efforts are ongoing to make this step more efficient, for example, by making a transformed cell line expressing the *Caenorhabditis elegans sid-1* that facilities dsRNA uptake through transmembrane channel formation (Xu *et al.*, 2013a,b) without the need for transfection reagents. Others have examined insect cells for the presence of RNAi associated factors, which helped with understanding of some of the inconsistencies reported when silencing genes in insect cell lines. This also led to transformed cell lines with enhanced RNAi capabilities including the *B. mori* and Sf9 cell lines described by Xu *et al.* (2013a,b)

and *B. mori* larvae and derived cell lines expressing *Bm-ago2* (Li *et al.*, 2015).

In terms of using RNAi technology to improve recombinant protein production, there are several advantages in deploying a system that is transient so that specific host or viral genes can be targeted at defined time points in the production process, especially where the gene to be silenced is essential for cell growth or virus vector replication. A good example of the use of RNAi technology to improve protein production is the silencing of apoptosis-related genes. Apoptosis, induced by nutrient limitation, shear or oxidative stress, or simply baculovirus infection, can lower the proportion of viable cells, leading to reduced yield and/or quality of target proteins. Although baculovirus-encoded anti-apoptotic genes like *p35* can inhibit the cellular response early in infection (Rohrmann, 2013), at later times it has been shown to be less effective (Lin *et al.*, 2007). Kim *et al.* (2007) showed that transfecting Sf9 cells with dsRNA against caspase-1 significantly decreased caspase-1 activity and since then others have produced transformed Sf9 (Lai *et al.*, 2012), *T. ni* High Five™ (Herbert *et al.*, 2009) and *B. mori* BmN (Wang *et al.*, 2016) cells that can stably down-regulate caspase-1 activity through RNAi. These studies reported that the cell lines were more resistant to apoptosis than the untransformed parental cell lines, enabling an extended window for protein production leading to higher yields – as much as 400% in some cases (Wang *et al.*, 2016).

When producing recombinant VLPs for early-stage vaccine studies, a common challenge is the unwanted co-purification of baculovirus particles that contaminate the final product. This is of concern to regulatory bodies that require VLPs to be free from contaminating proteins and/or nucleic acids. Lee *et al.* (2015) used RNAi to silence the baculovirus *gp64* that expresses the major virus glycoprotein GP64, which resulted in a significant, although not total, reduction in baculovirus particle formation. RNAi has also been used to silence viral cathepsin, whose presence may degrade recombinant proteins containing the appropriate target motif (Kim *et al.*, 2007); although use of an expression vector with *v-cath* (and viral *chitinase*) deleted from the virus genome (Hitchman *et al.*, 2010) is a more fool proof way of reducing proteolytic activity.

Engineering of insect cells to improve glycosylation of recombinant therapeutic proteins is an ongoing area of development for the baculovirus expression system. One approach has been to modify cell lines to express human glycosyltransferases, as described above. However, when these cells are inoculated with a virus expression vector, the shut-off of host cell genes means that as infection progresses, the supply of the required enzymes is reduced. An alternative approach, first described by Geisler *et al.* (2008), involves silencing of the gene (*fdl*) encoding N-acetylglucosaminidase, which very efficiently removes the terminal N-acetylglucosamine from the core oligosaccharide, thus preventing further elongation to form complex N-glycans. Whilst Geisler *et al.* (2008) used plasmids encoding an inverted repeat to down-regulate N-acetylglucosaminidase, more recent studies (Nagata *et al.*, 2013) have used RNAi to deplete the mRNA levels of the *Bmfdl* gene. In the latter study, almost all of the glycans synthesized were of the complex type.

Developments in cell lines to support recombinant protein production

Improvements and modifications to expression vectors involve just one half of the baculovirus-insect cell expression system. The other half comprises the host cell line, which in the past has received less attention than the virus vector. The most commonly used insect cell lines are derived from *T. ni*, the cabbage looper, or *S. frugiperda*, the fall armyworm. Most commercial products manufactured using the BEVS are made using the latter. The original cell line, IPLB-SF-21 (Sf21), was derived from pupal ovaries (Vaughan *et al.*, 1977) and the popular Sf9 cell line is a subclone of IPLB-SF-21. As discussed above, novel cell lines have been produced that have been engineered to improve recombinant protein quality and yield by either incorporating additional beneficial genes or deleting/silencing genes that may adversely affect protein production.

For recombinant products to be realized as commercial vaccines or therapeutic proteins, however, regulatory authorities require that the host cell is thoroughly characterized and, as far as is possible, shown to be free from adventitious agents that may contaminate the final product. Such studies led to

the discovery of a novel rhabdovirus in *S. frugiperda* cell lines (Ma *et al.*, 2014). This has raised potential safety concerns with the commercial production of proteins in insect cells, which led Maghodia *et al.* (2016) to develop an Sf cell line that was demonstrated to be rhabdovirus-negative (Sf-RVN). Sf-RVN has similar properties to Sf9 cells in terms of doubling time, diameter and morphology. Further, three different recombinant proteins were produced using both Sf9 and Sf-RVN and yields were indistinguishable between the two cell lines. Interestingly, titres of recombinant viruses reached higher levels (5- to 10-fold) in the rhabdovirus-free cell line and thus this cell line may prove to be an attractive alternative to Sf9 cells for the commercial manufacture of proteins.

Similarly, *T. ni*-derived High Five™ cells have been shown to contain a latent infection of a newly described alphanodavirus, which was discovered when the cells were being used to produce hepatitis E virus-like particles, which were contaminated with nodavirus particles (Li *et al.*, 2007). The sequence of the genome of the nodavirus was identical to that of the natural latent infection. Subsequently, a nodavirus-free cell line (Tnms42) was derived from T.ni High Five™ cells (Chen *et al.*, 2013).

While mammalian stable cell lines used for protein production have generally been well characterized regarding their transcriptional regulatory or secretory bottlenecks, relatively little is known about the stress and metabolic loads in insect cells. Using the alphanodavirus-free cell line Tnms42, Koczka *et al.* (2018) undertook a transcriptome analysis after infection with viruses expressing the recombinant proteins mCherry or influenza HA. Their analysis suggests that the main cellular response is to the stress caused by viral infection and secreted protein production (HA), which results in the up-regulation of host factors responsible for correct folding, disulphide bridge formation and glycosylation of proteins. Due to the class of proteins that were upregulated during cell stress, the authors suggest that these proteins are promising targets to be used in molecular engineering of insect cell lines to improve yields and overcome the problems of secreted protein production (as described above).

Linked with the development of new insect cell lines for recombinant protein synthesis by baculoviruses is the role that modifications to growth can play in determining productivity. The use of rapamycin and cysteine to enhance production of HCMV gB (Patrone *et al.*, 2014) has already been described. Monteiro *et al.* (2016) investigated the addition of different supplements to the insect cell medium to improve infectious BV and influenza VLP yields. Overall, the addition of cholesterol provided the main increase to both BV (2.5-fold) and VLP (6-fold) cell-specific yields. Other supplements such as reduced glutathione, antioxidants and polyamines increased the cell-specific yields of baculovirus particles up to 3-fold. The addition of both polyamines and cholesterol improved infectious BV production by preventing the accumulation of non-infectious particles. What these studies emphasize is that significant improvements to recombinant protein production may be achieved using simple modifications to the production process.

Future developments

The two original publications on the use of baculoviruses as expression vectors were significant milestones in demonstrating that both secreted eukaryotic proteins as well as bacterial products could be synthesized in insect cells (Smith *et al.*, 1983; Pennock *et al.*, 1984). These were later followed by the use of the silkworm baculovirus (BmNPV) as a convenient vehicle for protein production in whole insect larvae (Maeda *et al.*, 1985). The complete genome sequences of both AcMNPV and BmNPV were subsequently published and have provided valuable information on virus gene function for further development of baculovirus expression vectors (Ayres *et al.*, 1994; Gomi *et al.*, 1999, respectively). However, to date such modifications made to baculovirus expression have been relatively minor in their scope. The deletion or silencing of a few virus genes have helped improve protein production in some cases. Conversely, the addition of various genes that encode 'helper' functions such as chaperones have also been beneficial.

Another very significant milestone was the production of a synthetic baculovirus genome based on AcMNPV (Shang *et al.*, 2017). This virus (AcMNPV-WIV-Syn1) was assembled using a combination of PCR and transformation-associated recombination in yeast. It was fully infectious for both cell cultures and insect larvae. This technically

difficult feat opens the way to the design of minimal baculovirus replicons that might offer improved features as expression vectors. Baculovirus genes could be omitted or other genes added to optimize the synthesis of different recombinant proteins.

In this review, we have shown the advantages associated with various strategies for improvement of baculovirus expression vectors. What is apparent is how difficult it is to extrapolate results from one system to another. The challenge now is to draw the baculovirus expression vector modifications together in a rational way as well as paying attention to how these might be integrated with improvements in the host cells currently used for protein production.

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