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Genetic Engineering of Baculoviruses

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1. Introduction

Baculoviruses are arthropod-specific, enveloped viruses with circular, supercoiled double-stranded DNA genomes [1]. They infect *Lepidoptera* (butterflies and moths), *Hymenoptera* (sawflies) and *Diptera* (mosquitoes) [2]. While many viruses are studied because of their damaging effects, the study of baculoviruses was stimulated by their potential utility to control insect pests [3]. Later, the utility of baculovirus as gene expression vectors was evidenced and a new research area emerged [4]. A major step forward was the development of bacmid technology [5] (the construction of bacterial artificial chromosomes containing the genome of the baculovirus) which allows the manipulation of the baculovirus genome in bacteria. With this technology, foreign genes can be introduced into the bacmid by site- directed recombination or by transposition. Baculoviruses have been used to explore fundamental questions in molecular biology such as the nature of programmed cell-death [6]. Moreover, the ability of baculoviruses to transduce mammalian cells led to the consideration of their use as gene therapy and vaccine vectors. Strategies for genetic engineering of baculoviruses have been developed to meet the requirements of new application areas, and the establishment of new genetic modification systems is still necessary when an unexplored experimental system is to be addressed. The aim of this chapter is to detail the areas of application of the baculovirus in basic molecular biology and applied biotechnology and the strategies used to generate genetically modified baculoviruses according to each area of study.

1.1. Molecular biology of baculoviruses

Baculovirus genomes consist of a circular, double-stranded DNA molecule with a size ranging from 80 to over 180 kbp and encoding 90 to 180 genes.

The name "baculovirus" is derived from the latin "*baculum*" (stick), denoting the rod-shaped nucleocapsids that are 230– 385 nm in length and 40–60 nm in diameter [1]. The virions are enveloped and present two phenotypes: occluded virions (OVs) and budded virions (BVs). These two types of virions differ in the origin and composition of their envelopes and their functions in the virus life cycle. In both, the genome is complexed with multiple copies of a small basic protein (p 6.9) which neutralizes the negative charge of the DNA and this structure is protected by other proteins forming the nucleocapsid. The OVs are enclosed in a paracrystalline matrix forming occlusion bodies (OBs), which are orally infectious. Their morphology was initially used to define two major groups or genera of the Baculoviridae: the Nucleopolyhedrovirus (NPVs) and the Granulovirus (GVs). NPV OBs, called polyhedra, are about 0.6– 2 μm in size and their major occlusion protein is called polyhedrin. GV OBs, also known as granules or capsules, are oval-shaped with diameters in the range of 0.2–0.4 μm (Figure 1). OBs are highly stable and can resist most normal environmental conditions thereby allowing virions to remain infectious for very long periods of time.
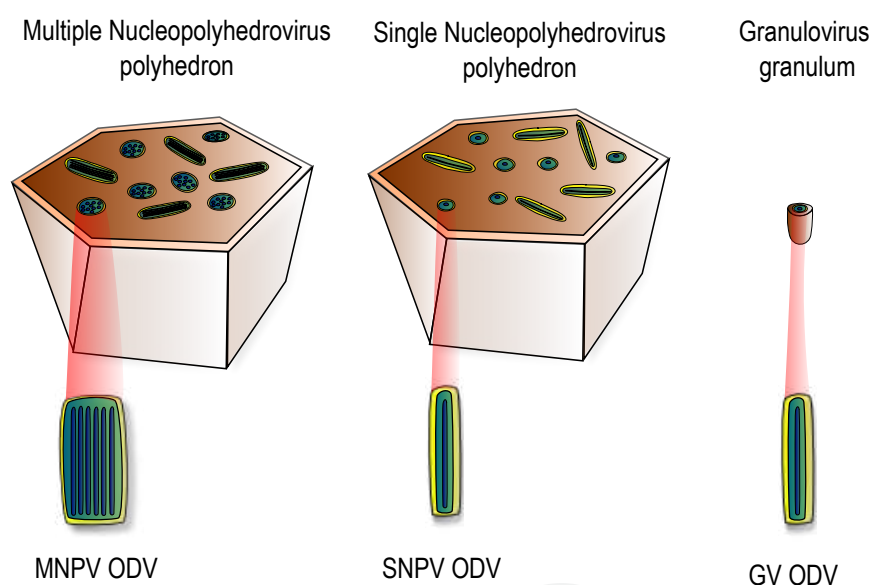

Figure 1. On the basis of the OB morphology, baculovirus were originally divided in two major groups: the Nucleopolyhedrovirus (NPVs) and the Granulovirus (GVs). NPVs occlusion bodies are called polyhedra and their major occlusion protein is called polyhedrin and GV occlusion bodies and granules or capsules for GVs.

Based on the fusion protein present in the BVs NPVs have been further classified into two groups: type I NPVs contain GP64, a low-pH-dependent membrane fusion protein required for virus entry and cell-to-cell transmission [7,8,9,10], BVs of group II NPVs and GVs lack a homolog of GP64, and membrane fusion during viral entry is triggered by F protein [11,12]. It was found that the entry of baculoviruses in mammalian cells is mediated by GP64. In contrast, baculoviruses with F protein cannot transduce these cells [13].

The natural cycle of infection by AcMNPV in insect larvae is summarized in Figure 2. Caterpillars ingest polyhedra that contaminate their food. The polyhedrin matrix is dissolved in the alkaline environment of the larvae midgut releasing ODVs (occlusion derived virions). These

virions enter midgut cells after fusion with membrane epithelial cells. The virions are uncoated and enter the nucleus where viral genes are expressed.

The gene transcription of baculovirus has been divided in sequential phases: immediate early, delayed early, late and very late. Immediate early genes are recognized by host transcription factors and viral proteins are not necessary at this stage. Transcription of delayed early genes requires activation by products of immediate early genes. The delayed early phase is followed by the synthesis of DNA and the late gene products of the virus [14]. There is a close relation-ship between the DNA replication and the switch to late gene transcription, and it is believed that these events are physically connected. In the late phase, that occurs following the initiation of viral DNA replication, nucleocapsid structural proteins are synthesized, including glyco-protein GP64 playing a crucial role in the horizontal infection by BV [15]. During the very late phase the production of infectious BV is greatly reduced. Nucleocapsids interact with nuclear membranes and eventually become enveloped usually in groups of a few particles. Envelop-ment of the nucleocapsids appears to be an essential primary step in the process of occlusion of nucleocapsids by the very late protein-polyhedrin. The occlusion continues until eventually the nucleus becomes filled with occlusion bodies. As occlusion proceeds, fibrillar structures begin to accumulate in the nucleus (sometimes also in the cytoplasm). These structures are composed mostly of a single polypeptide named p10, which is a very late protein [16]

The function of fibrillar structures is not clear but they may play a role in the controlled cellular disintegration in caterpillars [17,18]. In the terminal stages of infection two viral proteins, chitinase and cathepsin, act together to facilitate host cuticle breakdown [19]. After death the caterpillar liquefies and releases polyhedra which can infect other insects. At the end of the infection, OBs may account for over 30% of the dry weight of the larvae [20].

2. Baculovirus expression vectors

2.1. Introduction

The high levels of expression of the very late genes has been exploited to design the first vectors for foreign gene expression based on baculoviruses. They are especially suitable regarding safety (not harmful to non-target organisms) and easy containment in the laboratory. Baculo-virus vectors are used to infect insect cells or larvae where high levels of recombinant proteins are produced; the eukaryotic environment provides appropriate post-translational modifica-tions in comparison with prokaryotic expression systems. Insect cells to be used in the baculovirus expression system are derived from lepidopteran insects and are relatively easy to grow. No control of oxygen atmosphere is required. Moreover, insect cells can be adapted to serum-free media and production of recombinant protein can be scaled up to pilot plant or larger bioreactors [21, 22]. The lepidopteran insect cells used are also normally free of human pathogens. Thereby, the proteins produced in the baculovirus virus expression system can be used for functional studies, vaccine preparations or diagnostics.
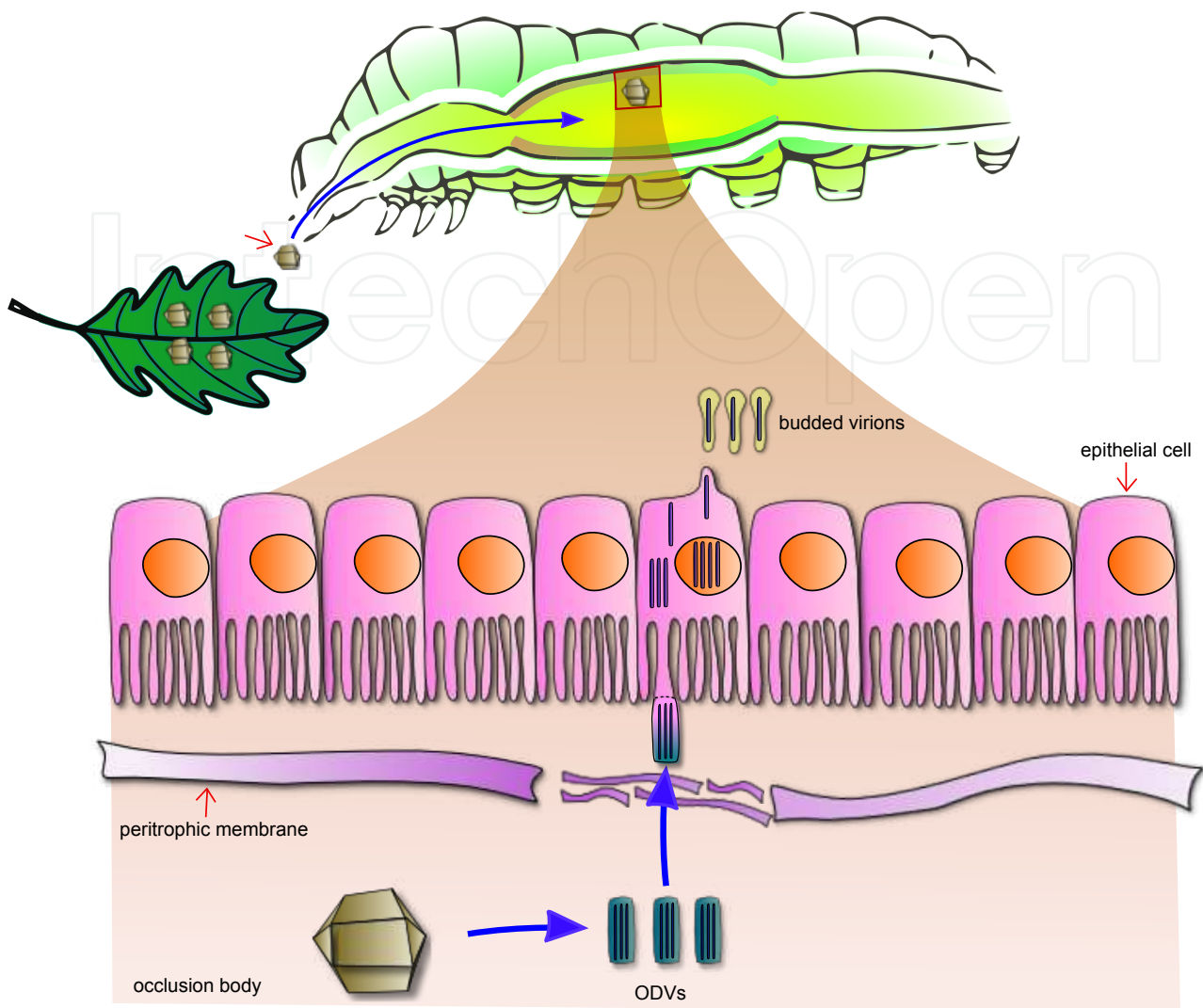
Figure 2. *Per os* infection of baculoviruses. A cross sectional representation of the anatomy of an insect larva is depicted. A baculovirus occlusion body (OB) is ingested in contaminated food. OBs pass through the foregut and enter the midgut where they dissolve in the alkaline midgut lumen and release occlusion derived viruses (ODVs).

A baculovirus expression vector (BEV) is a recombinant baculovirus that has been genetically modified to lead the expression of a foreign gene. BEVs are viable in insect cell culture and sometimes in larvae, depending on the baculovirus genes deleted in the process of the recombinant virus generation. In BEVs, the foreign gene coding sequence is usually placed under the transcriptional control of a viral promoter. For this reason usually viral factors are required for the transcription of the foreign gene.

The most commonly used process for cloning recombinant baculovirus is briefly summarized in Figure 3. Baculovirus genomic DNA and a transfer plasmid are cotransfected into an insect cell culture. Double homologous recombination between viral DNA and transfer plasmid causes the allelic replacement that incorporates the recombinant gene in the baculovirus

genome. Clonal purification requires several plaque passages. After this, viral stocks can be produced and amplified for recombinant protein production [31]. Insect cells are used for purification of many proteins, including therapeutic and vaccine peptides. Larvae are used to reduce production costs, or when recombinant baculovirus are to be tested as bioinsecticides. Finally, baculovirus can be used for transduction of mammalian cells, for production of therapeutic proteins, or to transduce organisms for gene therapy or vaccination.
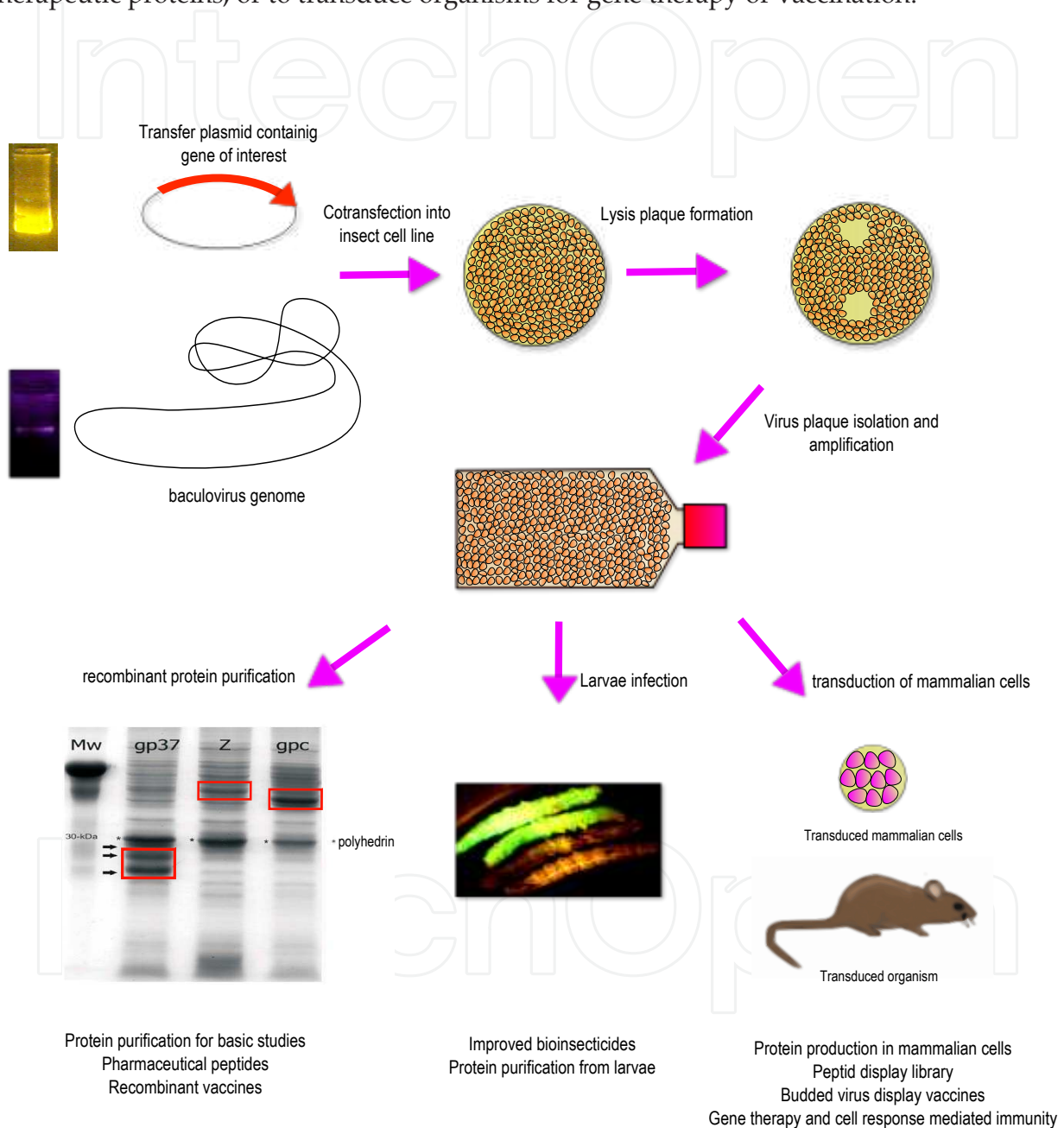

Figure 3. General process for baculovirus cloning. Genomic DNA and a transfer plasmid are cotransfected into an insect cell culture. Recombinant virus propagates causing lysis plaques. Virus is isolated from lysis plaques and amplified. Virus can be used for recombinant protein production in insect cells or larvae. Furthermore, baculovirus can be used for transduction of mammalian cells or whole animals.

2.2. Historical perspective

The major component of the polyhedrovirus OB is polyhedrin. This polypeptide comprises about 25% of total cell protein in the late phase of baculovirus infection [23]. This was the main property of baculovirus that led to their consideration as expression vectors. In the first studies of baculovirus, AcMNPV polyhedrin gene was located and cloned. Then, a plasmid containing the sequences of the polyhedrin gene (polh) and its flanking regions was constructed and subsequently the polyhedrin open reading frame (ORF) was replaced with the reporter gene β-galactosidase [24]. This is the simplest version of a transfer plasmid, which contains the strong polh promoter and upstream and downstream flanking sequences, but lacks the polyhedrin ORF which is usually replaced with the coding sequence of a foreign gene of interest. Transfer plasmid and viral DNA are cotransfected into cultured insect cells, where allelic replacement of polyhedrin can occur via homologous recombination involving the flanking viral sequences present in both DNAs.

This strategy was devised because the baculovirus genome is large [25]; and in vitro ligation of foreign DNA fragments with restriction enzyme-digested viral DNA has been successful only in few cases [26, 27]. Other strategies that have been explored include enzymatic recombination in vitro [28] and homologous recombination in yeast followed by selection [29]. Although these methods are ingenious, neither has become part of the mainstream baculovirus technology.

Allelic replacement is a consequence of double homologous recombination between viral DNA and transfer plasmid, and occurs at a frequency of only about 1% [30]. Thus, the viral progeny is a mixture of recombinant and wild type virus, which needs to be resolved in plaque assays. The recombinant progeny is occlusion negative (*occ-*) and produces polyhedrin- negative plaques (cell plaques with no polyhedral OBs), whereas wild-type progeny produces polyhedrin-positive plaques (*occ+*). Clonal purification requires several plaque passages. After this, viral stocks can be produced and amplified to infect cell cultures for recombinant protein production.

2.3. Strategies to simplify the isolation of recombinant baculoviruses

As mentioned before, following the strategy described above, the proportion of the progeny virus population derived from the cotransfection experiment is less than 1% [31]. Plaque purification of recombinant clones requires a tedious search for *occ-* plaques. Good quality microscope and experienced eye are necessary, and few recombinant viruses can be screened simultaneously because of the number of titrations required. To circumvent these problems, several modifications in the parental viral genome were carried out in order to simplify the isolation of recombinant baculoviruses, with the aim of reducing the parental virus yields in the progeny of the co-transfected insect cells.

One of the most successful strategies is the use of a linearized parental genome (in principle, no virus can be recovered) instead of the circular viral DNA (fully infectious). The addition of a unique naturally infrequent restriction site in the baculoviral genome allows the digestion with the adequate restriction enzyme and digested parental DNA is cotransfected with transfer

vector [32]. As linearized parental DNA has a reduced infectivity compared with its circular counterpart, frequencies of recombinant progeny rise to about 30%. The baculoviral genome can also be modified to contain restriction sites on both sides of a cassette containing a reporter gene coding sequence (such as β-galactosidase) under the control of a baculoviral promoter, so that double digestion with this enzyme removes the reporter gene cassette. The presence of two restriction sites reduces the frequency of undigested circular DNA genome. Moreover, if undigested or single digested-repaired parental DNA produces progeny, those few parental plaques stain blue in the presence of X-gal and can be easily discarded. This strategy was exploited in the AcMNPV BEV system. In this virus, the genome was modified to contain Bsu36I sites on both sides of the β-galactosidase sequences, so that digestion with this enzyme removed the gene and also part of a virus gene (ORF 1629) that encodes a structural protein [33]. By removing part of the essential ORF 1629 gene, the virus is unable to form infectious particles efficiently even if the double digested linear DNA is repaired and recircularized in insect cells. In contrast, a process of homologous recombination repairs the deletion of ORF 1629 while simultaneously inserting the foreign gene in place of β-galactosidase. Several commercial systems make use of the repair of the deletion in ORF 1629. Among them, Bac-to-Bac® (Invitrogen) and flashBacTM (Oxford Expression Technologies) quickly gained popularity in the scientific community.

2.4. Bacmid technology

A major step forward in the technology of baculovirus genetic engineering has been the development of baculovirus genomes capable of replicating in a bacterial host as bacterial artificial chromosomes (Figure 4). These recombinant baculoviruses are called bacmids, and they have been modified to contain classical bacterial artificial chromosomes replicons and selection markers for selection in bacteria. BAC vectors contain a fragment of *E. coli* fertility factor (F- factor) replicon (*miniF*) and are maintained as circular supercoiled extrachromosomal single copy plasmid in the bacterial host [57, 58]. BACs can accept inserts up to 300 Kb in length. The principal advantage BACs have over other high insert capacity vectors like yeast artificial chromosomes (YAC) and mammalian artificial chromosomes is stability of insert propagation over multiple generations.

Once transferred into the bacterial host, the baculovirus genome can be manipulated easily through site-specific recombination, Rec-A mediated homologous recombination or transposition. Once the recombinant bacmid is generated and the presence of transgene and the absence of the parental bacmid in the bacterial colonies are verified, e.g., by PCR, the DNA from those colonies is purified and used to transfect susceptible insect cells. As was mentioned above, naked genomic DNA from baculovirus can efficiently establish infection when it reaches the cell nuclei. BV particles can be recovered from culture supernatant and used as inoculum to produce high titer stocks.

Various commercial transfer vectors are available and compatible with bacmid systems to allow expression of one or two proteins (*e.g.*, pFastBac1TM and pFastBacDualTM from InvitrogenTM). Some are designed to add tags and signal peptide sequences fused to the

protein of interest in order to facilitate their purification. In addition, transfer vectors that are compatible with GatewayTM and TOPO® cloning technologies have been developed.

A problem frequently found when working with BAC systems is the presence of parental bacmid background even in the same colony where the recombinant bacmid is found, despite the antibiotic-based selection and blue-white screening. This then requires a new transformation of bacterial cells with mixed DNA and the screening of newly replated colonies. To avoid this requirement, a negative-selection system has been developed that makes use of the *sacB* marker [59]. When the transposon is not integrated in the bacmid genome, the bacterial cell will be killed in presence of sucrose due to the expression of *sacB* gene, which encodes an enzyme that metabolizes sucrose to a toxic compound.

The first bacmid developed contained the AcMNPV genome. Later, bacmid systems were developed for *Bombyx mori* NPV, *Helicoverpa armigera* single-nucleocapsid nucleopolyhedro-virus *(HearSNPV)* [61] and *Cydia pomonella* granulovirus (CpGV) [60] (the first report of a granulovirus bacmid).

Later, bacmid technology was exploited to develop a system that allows the generation of recombinant baculoviruses with negligible background. This system relies on homologous recombination in insect cells between a transfer vector containing a gene to be expressed and a replication-deficient AcMNPV bacmid. The deficiency of AcMNPV is due to a deletion in the essential gene *orf1629*, and homologous recombination between bacmid DNA and transfer vector (containing *orf1629*) repairs this deleted gene [62, 63, 64]. Therefore, only recombinant virus can replicate and no further selection is required, facilitating the rapid production of multiple recombinant viruses on automated platforms in a one-step procedure. Several commercial vectors (flashbackTM, Oxford Expression Technologies Ltd., BacMagicTM (Merck), BaculoOneTM (PAA), etc.) follows this principle. All these systems claim that no plaque purification of baculovirus is required, although it is recommended (there is a possibility that defective genomes can be replicated when a replication competent viral genome resides in the same cell). Later, bacmids using this selection system were improved for protein expression, carrying additional deletions in cathepsin (*v-cath*) and p10 gene [65].

2.5. Improving protein quality and quantity in baculovirus expression systems

2.5.1. Introduction

The proteins to be expressed using recombinant baculovirus and insect cells are commonly of mammalian origin and, as it happens in other expression systems, the expression levels and the conformation and posttranslational modifications vary among individual proteins. The principal purpose of a protein expression system is not only the production of large quantities of recombinant protein, but also the production of a recombinant protein that resembles the native protein. One of the most difficult challenges in expression systems is the expression of transmembrane proteins. The correct expression of complex transmembrane proteins that cross the membrane several times is even more difficult. In order to improve the quality in the routing, the post-translational modifications and the stability of recombinant proteins, several modifications have been carried out that address these limitations of baculovirus expression
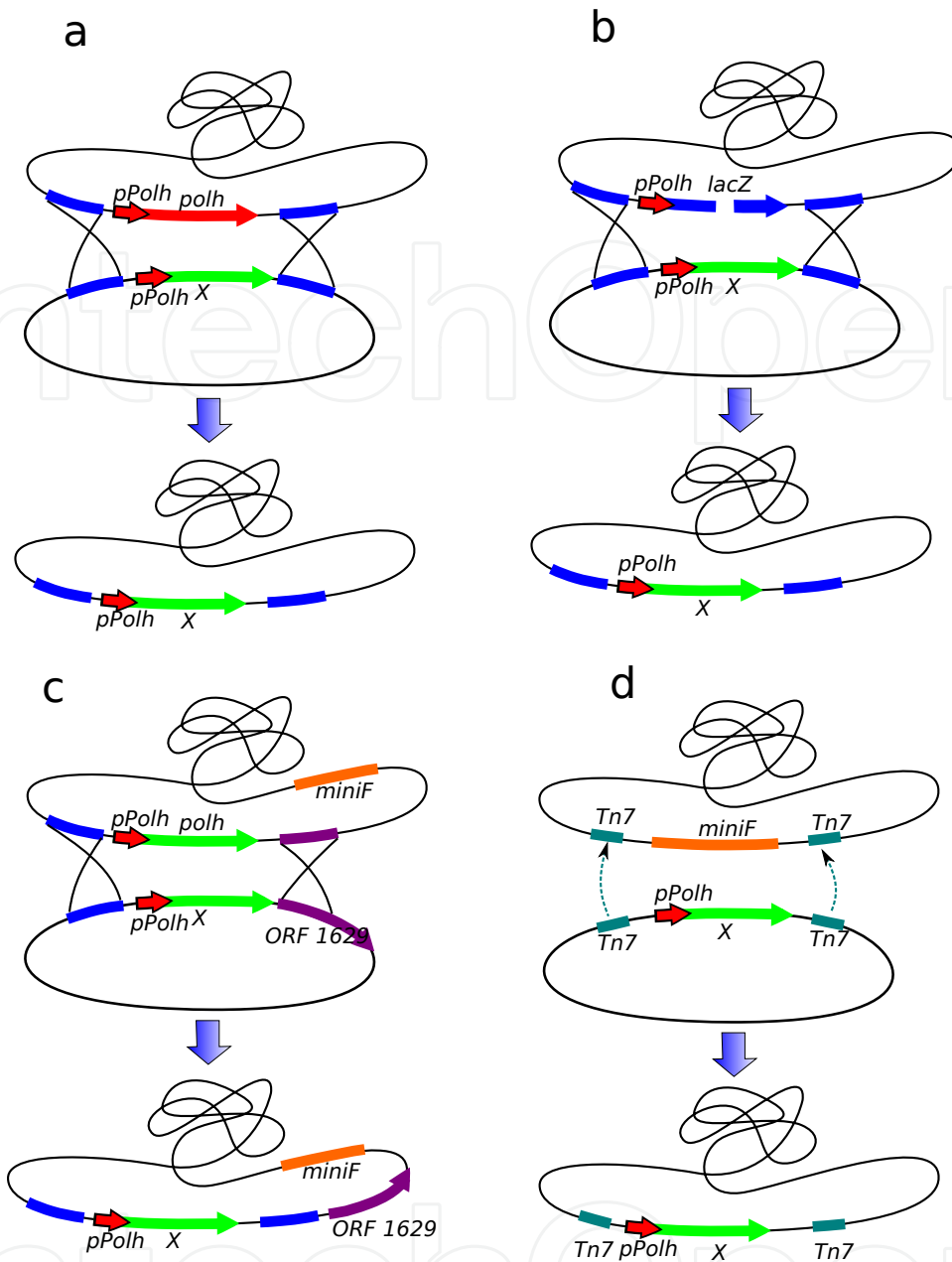
Figure 4. Baculovirus expression vectors over time. Various methods exist to generate recombinant baculoviruses expressing a foreign gene (gene X). Historically, recombinant baculoviruses were generated through homologous recombination (1). Subsequently, linearized vectors were developed to increase the percentage of recombinants (2). Bacmid technology allowed the maintenance of defective baculoviruses as bacterial artificial chromosomes. Homologous recombination with transfer vector in cells repairs the essential gene (3). Bacmid technology also allowed the generation of recombinant baculovirus by in vitro transposition (4).

system. It is important to notice, however, that the protein is produced in the context of a viral infection. Since certain protein processing pathways are compromised by baculovirus infection, the capacity of host cells to correctly route, fold and modify the recombinant protein is affected. This intrinsic limitation must be recognized and baculovirus expression system must be regarded as a transient expression system.

2.5.2. Heterologous DNA properties and codon usage

Although the promoter elements that control the transcription of the heterologous gene are derived from baculovirus, it is important to consider the effect of introducing heterologous or artificial 5′ and 3′untranslated regions (UTR). The 5′UTRs of baculovirus are short AT-rich sequences. Therefore, the introduction of GC-rich sequences upstream of the ORF may have a negative effect on the heterologous gene transcriptional levels. The choice of 3′UTR, including polyA sequences may also determine the heterologous gene expression levels. As expected, p10 polyA signals are more efficient than the widely used SV40 terminator [66]. There are no in-depth studies comparing the influence of codon usage on translation levels in baculovirus expression systems. However, no strong bias in alternative codon frequency has been observed in baculovirus coding sequences, suggesting that the codon optimization is unlikely to improve significantly the translation levels [67].

2.5.3. Deletion of baculovirus genes to prevent proteolytic cleavage

Chitinase, the product of the gene *chiA*, is an enzyme that breaks down the chitin exoskeleton of the insect host, together with cathepsin (V-CATH, encoded by *v-cath*) at the end of the infection, ensuring the dispersal of the viral occlusion bodies [68]. As those genes have a specific function in the context of the infection in the insect, they are not required for the propagation of the virus in cultured insect cells. Chitinase is produced at high levels and stored in the endoplasmic reticulum, and it may interfere in the secretory apparatus of the host cell. On the other hand, cathepsin is a protease that is made as an inactive precursor (PRO-V-CATH). PRO-V-CATH can be activated when preparing protein samples for SDS-PAGE, leading to the degradation of the recombinant protein. Bacmids were developed with deletions in the genes *chiA* and *v- cath*, resulting in higher levels of secreted protein [69, 70].

2.5.4. Secretion of proteins

Many secretory pathway proteins have N-terminal signal peptides that direct the protein correctly through the ER and the Golgi system and ultimately to the surface of the cell. If the signal peptide is not adequately recognized, the protein may be not targeted to the cell surface, and the misfolded protein is also prone to degradation as it may be recognized by quality control systems [71].

Native signal peptides of mammalian proteins may be replaced by signal peptides derived from insect proteins such as the signal peptide of honey bee melittin [72] or derived from baculovirus proteins, such as the GP64 signal peptide. Although the introduction of insect signal peptides normally targets the protein to cell surface, it does not always lead to a correct folding of the protein.

2.5.5. Glycosylation of proteins in the baculovirus-insect cell system

Glycosylation is a common covalent chemical modification that can affect many protein properties, including intracellular trafficking, biological function, immunological properties and biochemical stability. One of the most advantageous features of the baculovirus-insect cell

system is that it can produce glycosylated proteins. However, the protein glycosylation pathways of lepidopteran cells differ from those of higher eukaryotes [73]. N-glycosylation begins in insect cells with the transfer of the oligosaccharide Glc3Man3GlcNAc2 (where Glc, Man and GlcNAc refer to glucose, mannose and N-acetylglucosamine, respectively) from a lipid complex to an asparagine residue in the polypeptide chain in the ER lumen. As the protein passes through the ER and Golgi system, enzymes trim and add different sugar moieties to this N-linked glycan. In this step is where insect and mammalian cells start to vary. This results in glycoproteins with simple oligomannose sugar chains in insects, while in mammals complex sugar groups with terminal sialic acids are added.

Differences in glycosylation patterns may affect the folding and targeting of recombinant glycoproteins and their immunological properties. Moreover, differences in glycosylation may even lead to protein degradation [74].

To overcome the limitations of the baculovirus insect-cell system in glycosylation, a series of transgenic cell lines derived from lepidopteran Sf9 and High Five cells expressing genes for the enzymes required to produce the complex mammalian glycosylation patterns were developed [75, 76]. Genes incorporated include bovine β-1,4-galactosyl transferase and rat α-2,6 sialyltransferase. The introduction of these enzyme resulted in the incorporation of galactosyl and sialyl residues in the produced proteins.

2.5.6. Expression of cytosolic and ER processing enzymes

Proteins to be secreted are translated in the cytosol and can translocate across the ER membrane either by a cotraslational or post-traslational mechanism. Transport in mammalian cells is primarily cotraslational, and in yeast both post-traslational and cotraslational mechanisms are used. In insect cells, the predominant mechanism is still not known. In mammalian cells, the cytosolic chaperone hsp70 is believed to contribute to the traslocation of proteins by interacting with nascent polypeptides and preventing their aggregation. Coexpression of immunoglobulin G (IgG) and human hsp70 resulted in higher levels of soluble IgG precursor. As a consequence, mature IgG secreted levels increased [77].

In the ER chaperones also assist the folding of polypeptides by preventing improper aggregations and conformations. In mammalian cells, immunoglobulin heavy chain binding protein (BiP) is an ER chaperone that interacts with several polypeptides destined for secretion and may be involved in the translocation or proteins across the ER membrane. When recombinant BiP was coexpressed in insect cells with IgG the soluble and secreted IgG levels were increased [78]. Other additional chaperones, such as calnexin and calreticulin, can also assist folding and assembly of membrane proteins in BEVs. Catalytic enzymes in the ER also collaborate by accelerating the folding. Disulfide bond formation occurs in the oxidizing ER compartment, with the catalytic action of protein disulfide isomerase (PDI). Studies demonstrated that overexpression of PDI increases the folding and secretion of IgG in insect cells [79]. It has been observed that co-expression of foldases appears to work more efficiently when the corresponding genes are provided by the baculovirus vector than those integrated in the genome of transgenic cell lines. This observation may be related to the phenomenon of host genome transcriptional shut down known to occur during baculovirus infection.

2.6. Improving baculovirus genome stability

A major drawback that limits the application of baculovirus for large-scale production is the accumulation of defective interfering (DI) particles upon serial viral cell culture passages. DI particles are not able to propagate autonomously due to deletion of large portions of their genomic DNA, but can co-propagate in the presence of viable virus [80]. Since deletion often includes the inserted foreign gene of interest, when DI particles proportion increases, re-combinant protein expression levels decreases. The accumulation of DI particles can be reduced by the practice of infecting at low multiplicities of infection (MOI).

Genetic engineering strategies have been developed to prevent the accumulation of DI particles. In *Spodoptera exigua* MNPV (SeMNPV), it has been observed that DI particles are enriched in a non-*hr ori* fragment. Removal of this *non-hr ori* from the genome of the baculovirus prevented the formation of DI particles up to 20 cell culture passages [81]. Removal of an AcMNPV *non- hr ori* had the same effects on genomic stability. It was also observed that when a large foreign fragment of DNA is cloned in baculovirus DNA genome in which no selection pressure exists, the addition of an *hr* (which functions as origin of replication) may prevent the loss of the foreign DNA [82].

3. Baculoviruses as bioinsecticides

3.1. Introduction

The basis of modern baculovirology was stimulated by the potential utility of baculoviruses to control insect pests [34]. Baculoviruses are highly infectious and selective pathogens (their host range is usually limited to one species), are very safe to people and wildlife and long term crop protection can be established [35]. Despite these advantageous features, the application of baculovirus as bioinsecticides has not still matched their potential. Although the use of baculovirus bioinsecticides was hampered by their slow speed of action when compared with fast-killing chemical insecticides, they gained increasing acceptance as they were considered for long term protection of crops, in the framework of integrated pest management.

Up to date, the most successful project was implemented in Brazil where over two million hectares of soybean were controlled by baculovirus AgMNPV [36, 37]. However, it is important to notice that a series of factors contributed to the success of AgMNPV as bioinsecticide. First, AgMNPV is highly pathogenic and only one application is sufficient to control the pest over the production cycle. In second place, *Anticarsia gemmatalis* was the most important plague in soybean crops in Brazil, and other plagues did not cause significant economic damage. Finally, the application of AgMNPV was promoted by Brazilian state and the integrated pest man-agement governmental programs facilitated the public acceptance of alternatives to chemical insecticides. Despite this favorable unique context, the success of Brazilian project revitalized the interest in baculovirus as bioinsecticides and many countries and private companies begun to develop new programs of baculovirus control and the search of novel baculoviruses.

3.2. Genetic improvement of baculovirus insecticides

In the search of increasing the commercial fitness of baculovirus as bioinsecticides, strategies to improve the baculovirus pesticide parameters by means of genetic engineering were developed.

Slow action of baculoviruses often limits its practical application and many strategies aimed to improving the timing of the pest killing or paralyzation by baculovirus. The first strategies were based on the interference of host physiology with insect hormones. When a diuretic hormone gene was introduced into *B. mori* baculovirus genome, recombinant BmNPV killed larvae about 20% faster than wild type virus [38]. The expression of this hormone by baculovirus causes the infected larvae to rapidly lose water.

Another strategy was based upon the control of juvenile hormone. In lepidoptera, this hormone controls the onset of metamorphosis at the final molt. The expression of juvenile hormone esterase decreases the concentration of the hormone [39, 40]. A reduction in the levels of juvenile hormone (JH) early in the last larval instar has been shown to initiate metamorphosis and lead to a cessation of feeding behavior. If this juvenile hormone esterase (JHE) is inhibited, the concentration of JH remains high enough to keep the larva in the feeding stage, resulting in giant insects. Another approach used consists in the deletion of the virus-encoded ecdysteroid glucosyltransferase gene [41]. The product of the *egt* gene normally prevents larval molting during infection increasing feeding activity of infected larvae. The EGT enzyme inactivates hormone ecdysone by transferring sugar molecules. The inactivation of this hormone results in an increased food consumption, allowing the virus to maximize the viral progeny. The infection with an *egt* defective recombinant AcMNPV resulted in a 30% faster killing of larvae and significant reduction in food consumption.

The degree of improvement that can be achieved by gene deletion alone appears limited. For this reason, several research lines have focused on the use of gene insertion technology in order to achieve more substantial improvement in the performance of viral insecticides.

Among the strategies that have been explored to date, the insertion of insect-specific toxins is the most promising one for development of commercially viable baculovirus insecticides [42]. In nature, insect predators and parasites use venoms to immobilize their prey. Although arthropod venoms are composed of a mixture of toxins that may have activity against organisms other than insects, it is possible to isolate genes that target insects with high specificity.

Although the first experiments using an insect-specific toxin of the scorpion *Buthus eupeus* [42] did not show an improvement in the speed of action of the recombinant baculovirus, the use of other scorpion toxin genes resulted in significant enhancement of virus insecticidal performance. One of the most promising insect-specific toxins used for the generation of recombinant baculovirus is the product of the gene AaIT of the scorpion *Androctonus australis*. The product of this gene is a small peptide (70 amino acids) that interacts with voltage- dependent sodium channels causing rapid paralysis in insects. Moreover, AaIT has no activity on vertebrate nervous tissue and is nontoxic to mice. When AaIT toxin was introduced into

AcMNPV, the speed of kill increased by about 40% and the feeding damage was reduced by about 60% [43].

Another paralytic toxin that holds promise is the TxP-I toxin, a component of the venom of the predatory straw itch mite *Pyemotes tritici* [44,45]. The mechanism of action this toxin has not been studied in depth, although it is related to voltage-dependent calcium channels (VDCC). The mean time to death of larvae infected with AcMNPV recombinant baculovirus expressing TxP-I under the control of p10 very late promoter was reduced by 50-60% compared to larvae infected with the wild-type strain, depending on virus dose and larval instar [46].

The choice of the promoter that controls the transcription of the heterologous toxic gene is very important. Although *polh* and *p10* very late promotes provides high levels of transcription, early and late viral promoters or constitutive promoters can result in an earlier accumulation of the toxin, causing more significant reductions in the speed of paralysis of the larvae. A chimeric promoter constructed by insertion of the p6.9 promoter downstream of the *polh* promoter was found to be more effective than *polh* promoter alone [47]. Another promoter tested was the constitutive *Drosophila hsp70* heat-shock protein gene promoter [48]. Despite the lower levels of toxin accumulation, the results obtained with this promoter were comparable to those obtained with the p6.9 promoter. The choice of the promoter must be considered from a biosafety perspective. Evidence indicates that recombinant baculoviruses expressing toxin genes are not pathogenic to vertebrates, and that the probability of horizontal transfer of the toxin gene to vertebrates is very low. Moreover, as it was mentioned above, specific arthropod toxins have no effect on vertebrate neural system. Despite these arguments, it is desirable to select promoters that are not functional in vertebrates.

3.3. Strategies for modifying host range of baculoviruses

A primary advantage of baculovirus bioinsecticides is their host specificity. In contrast to chemical insecticides that may harm vertebrates or kill arthropods indiscriminately, baculoviruses target specific populations of insect pests. This feature makes them compatible with classical biological controls in integrated pest management strategies and makes particularly useful for controlling insect pests in environmentally sensitive areas. Although bioinsecticides are attractive from an ecological perspective, their limited host range is undesirable from an economical point of view. Since many different baculoviruses may be needed to control complexes of simultaneous insect pests, costs would be excessively high. For this reason, many researchers have studied the possibility of modifying the baculovirus host range while maintaining their safety for vertebrates and nontarget arthropods.

3.4. Determinants of virus host range

The host range of any virus is determined by its ability to enter the cells and tissues of a host organism, replicate and release new infectious virus particles. The virus host range is frequently determined by the presence of suitable receptors that facilitate virus attachment and entry into a host cell. This does not appear to be the case for baculoviruses. Baculoviruses are able to enter nonpermissive insect and even mammalian cells. This indicates that if receptors are used by baculoviruses, they are common to insect and mammalian cells [49, 50, 51, 52]. In

nonpermissive insect cells, reporter gene expression was observed from early baculovirus promoters, but expression from very late baculovirus promoters was limited. Expression from late baculovirus promoters varied among nonpermissive insect cell lines. As mentioned before, it was established that the transcription from late baculovirus promoters requires the viral DNA replication [53]. These findings indicate that in nonpermissive insect cells viral DNA is delivered to the nucleus, the site of baculovirus replication, although replication is restricted in a cell specific manner. For this reason, the viral genes that determine the host range are likely to be related with the process of DNA replication.

One of the first steps forward in baculovirus host range alteration was the generation of a recombinant AcMNPV capable of replicate in nonpermissive *B. mori* cells and larvae. This was achieved by replacing the endogenous *p143* gene, which encodes an essential protein with homology to DNA helicases by a hybrid *p143* gene [54]. The hybrid *p143* gene resulted from the homologous recombination between AcMNPV and BmNPV *p143* genes, and differed from AcMNPV *p143* only in four amino acids. How these changes in *p143* affected AcMNPV host range is still not well understood. Infection of *B. mori* BmN cells by wild type AcMNPV induces protein synthesis arrest [55]. This suggests that AcMNPV *p143* or perturbations in the cell caused by AcMNPV *p143* or its activity may induce a cellular response. This example demonstrates that baculovirus host range can be manipulated through genetic engineering. However, it is important to notice that BmNPV and AcMNPV are closely related baculoviruses showing on average ORF amino acid sequence identities of about 93%. Although deletion of a gene critical for replication in one host can reduce the virus host range, in many cases, the insertion or modification of a single gene will not be sufficient to expand host range. The expanded AcMNPV host range resulting from *p143* recombination with BmNPV is probably a singular case. Functional complementation studies have conducted to the identification of other viral elements that may result in host- specific interaction. Those elements include the homologous regions (*hrs*). *Hrs* consist of repeated units of about 70 base pairs with an imperfect 30 base pairs palindrome near their center, and have been implicated both as transcriptional enhancers and origins of DNA replication for a number of baculoviruses. It was demonstrated that *hrs* interact with host and viral factors in a species-specific way. In an interesting work baculoviruses were analyzed by bioinformatics in the search of genes subject to positive selection pressure (when the rate of nonsynonymous substitutions per potential nonsynonymous site in a gene is greater than the rate of synonymous substitutions per potential synonymous site, the gene is said to be undergoing positive selection). Since most genes appear to be subject to negative selection most of the time, this method can be used to identify viral genes involved in adapting to new or current hosts [56].

Another relevant topic to be addressed in the development of baculovirus recombinants with expanded host range is the selection of appropriate promoters for the expression of heterologous genes. If selected candidate genes for expansion of the host range are to be incorporated in the baculovirus genome under the control of their own promoters, it is necessary to evaluate the functionality of these promoters in this context.

4. Mammalian cells transduction and BacMam systems

4.1. Introduction

Initial interest in baculoviruses as gene delivery vectors for mammalian cells was driven by their good biosafety profile [84]. Compared to other human-derived viral gene delivery vectors, the safety requirements for handling baculoviruses are relatively low. Baculoviruses are so exceptionally adapted to their natural hosts that they pose no threat to vertebrate organisms. They are unable to replicate in mammalian cells, can be manipulated in laboratories at BSL1/2 levels and can be easily inactivated [85]. Moreover, insect larvae in the wild are infected via the gut by occluded baculoviruses and polyhedrin-deleted recombinant virus used to transduce mammalian cells does not efficiently infect larvae. The viruses are unstable outside of the laboratory, so they are environmentally contained as well.

Baculovirus entry into mammalian cells was suggested to depend on electrostatic interactions, heparin sulfate and phospholipids, but the exact cell surface molecules for baculovirus docking remained unknown [86]. It was also proposed that clathrin-mediated endocytosis and macropinocytosis play roles in baculovirus entry [87, 88]. Contradictorily, a recent study [89] discovered that (1) baculovirus entered cells into vesicles devoid of clathrin; (2) macropino-cytosis-related regulators imparted no significant effects on virus transduction and (3) the internalization and nuclear uptake were affected by the regulators of clathrin-independent entry. These data unveiled a baculovirus entry pathway independent of clathrin-mediated endocytosis and macropinocytosis and suggested that phagocytosis might play a role, which echoed the observations reported previously [90]. Moreover, other recent studies reported that baculovirus transduction related to direct fusion pathway induced by a short pH trigger [91]. Nevertheless, one consensus is that baculovirus envelope protein gp64 is pivotal for entry because blocking gp64 can abrogate the baculovirus ability to transduce mammalian cells and activate dendritic cells [92]. Very recently, it has been demonstrated that 6-O- and N-sulfated syndecan-1 promotes baculovirus binding and entry into mammalian cells. [93].

Numerous cell lines have been transduced [94], including primary cells in vitro and human livers ex vivo and the capability of baculovirus as a gene therapy vector has been studied. More recent studies have described the use of AcMNPV vectors in the form of BVs for in vivo targeting of different organs including brain and liver [95], and stem cells for tissue engineering [96].

The term BacMam refers to baculoviruses in which a mammalian promoter is used to drive heterologous gene expression in mammalian cells following viral transduction (Figure 5). Since the viral genome can stably accommodate an insert sequence of at least 40 kb, BacMams are particularly suitable for expression of multimeric complexes. Unless a selection force is applied, gene expression in transduced cells is transient and can usually last for up to 4 days. However, the expression can even be prolonged to 16 days. For viruses carrying a selectable marker, stable cell lines can also be established upon selection [97].

BacMams have been used as delivery vehicles to mammalian cells for many polypeptide genes, including secreted [98] and transmembrane proteins [99, 100, 101]. When high MOIs are used,
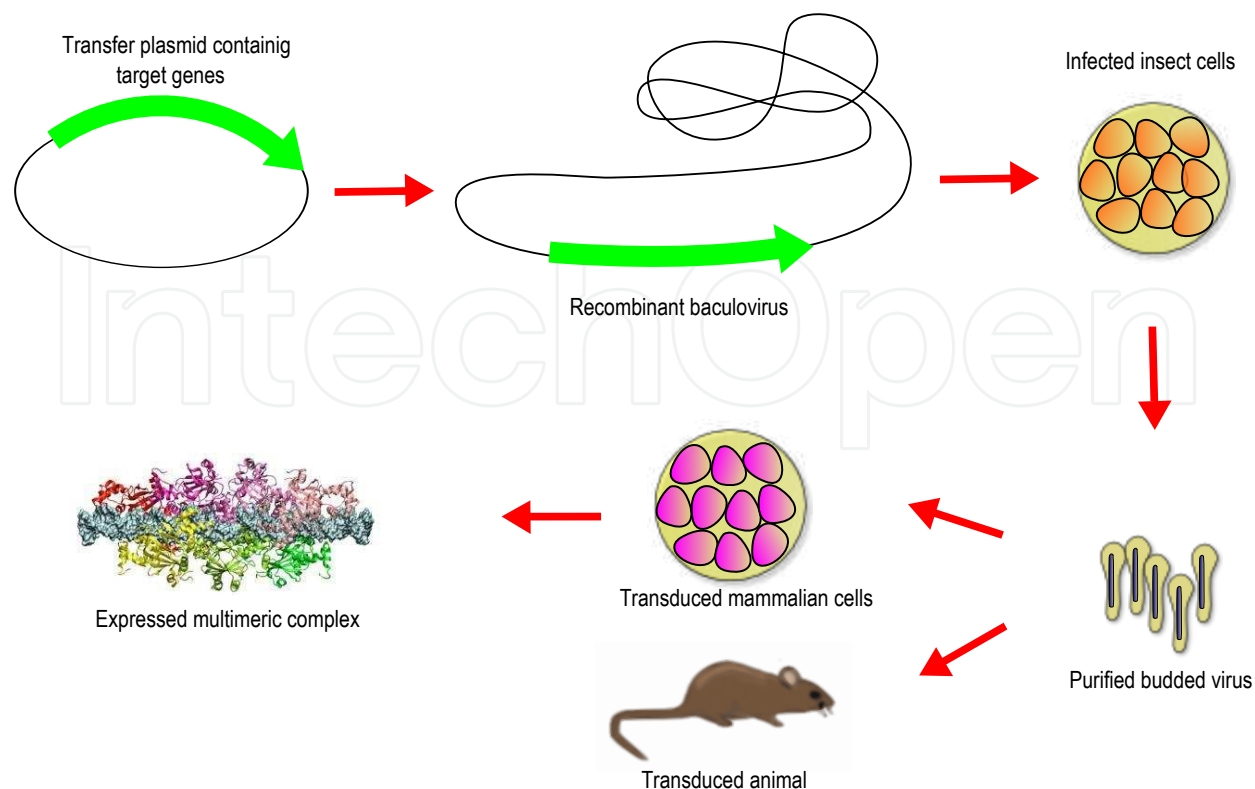
Figure 5. Production and application of BacMam viruses. Target gene sequences cloned into a transfer plasmid containing a mammalian cell-active expression cassette are transferred to baculovirus DNA via recombination. The viral DNA is transfected into insect cells where virus production occurs. Budded virus are clarified from the insect cell culture medium. The stock virus is used to transduce mammalian cells and expression of the recombinant protein(s) is usually validated 24–48 h later. The effects of the expression can be tested with this transient assay. BacMam vectors can be also used to transduce mammalian organisms.

transduction efficiencies near 100% can be reached. With this high transduction efficiency and flexibility, the technology easily enables coexpression of several genes with multiple baculoviruses and modulation of expression level by dosing and timing. This flexibility is especially relevant in studies of multimeric complex functional proteins and also in assays of processes where mix-and-match coexpression experiments with a number of cofactors and interacting partners are necessary.

4.2. Available vectors for BacMam development

The vectors used for the development of BacMams are derivatives from AcMNPV transfer vectors. The most widely used system for the generations of BacMam are based on the Bac-to-Bac system (InvitrogenTM) for baculovirus generation. With this system the recombinant baculoviral genome is constructed in *E. coli*, via a transfer vector. The gene of interest is first subcloned into a BacMam transfer vector, which is then transformed into a special *E. coli* strain DH10Bac to generate the recombinant viral DNA. The viral DNA is then used to transfect insect cells in order to generate the recombinant virus. The entire process is simple and easy to perform, allowing generation of multiple viruses simultaneously. With the procedure, recombinant BacMams can be generated in less than 2 weeks. The Bac-to-Bac system [102] uses

the Tn7-mediated site-specific transposition reaction to direct integration of expression cassettes contained in the transfer vector into a baculovirus backbone vector (bacmid) preexisting in the *E. coli* DH10Bac strain. In this case, the bacmid is a mini-F replicon with the baculovirus genome and has a kanamycin resistance marker. In addition, the *E. coli* strain contains a helper plasmid that expresses the Tn7 transposase gene. The system was designed in such a way that the recombinant Tn7 transposon from the transfer vector will be integrated into a mini-attTn7 in the *lacZα* gene fragment contained within the recombinant viral genome, causing inactivation of the α-complementation of *lacZ*. The desired recombinant transformants will be resistant to tetracycline, kanamycin, and gentamicin and can be easily distinguished from nonrecombinants by blue/white selection on X-gal plates.

The BacMam transfer vectors described here are derivatives of pFastBac1 of the Bac-to-Bac system (InvitrogenTM). Originally, the AcMNPV *polh* promoter of pFastBac1 was deleted for the introduction of cassettes containing a mammalian promoter. Later, the CMV immediate early promoter was inserted to allow expression of the cloned cDNA sequences in mammalian cells. The vector pFastBacMam-1, in addition, contains a neomycin resistance gene driven by the SV40 promoter. The neomycin resistance marker allows selection of stable cell lines following BacMam transduction. Using this vector a new version was constructed (pFast-Backmam-NA) to accommodate ORFs cloned in GatewayTM vectors (Invitrogen).

4.3 Strategies to improve baculovirus transduction

4.3.1. Surface display via gp64 fusion or expression of heterologous protein

Heterologous peptides can be inserted between the signal peptide and the mature domain of the envelope fusion protein GP64, and this feature has been exploited for surface display of peptides to improve the virus transduction [103, 104], for ligand-directed targeting if an appropriate ligand is chosen [105, 106]. When a short peptide motif from gp350/220 of Epstein–Barr virus (EBV, which naturally infects B cells) was displayed as GP64 fusion peptide on the baculovirus envelope [107], the efficiency of transduction to B lymphocytes was increased. Another paradigm is the display of the immunoglobulin Fc region on the baculovirus surface [108]. Fc receptors (FcRs) are membrane proteins that bind to the Fc region of antibody and mediate the phagocytosis and antigen presentation. The Fc display allows for specific baculovirus targeting to cell lines and antigen presenting cells (APCs) expressing FcRs, hence augmenting the vaccine effect. The display system also allows for the surface presentation of functional membrane proteins to simplify subsequent isolation.

Aside from the gp64-aided display, expression of vesicular stomatitis virus G protein (VSVG) [109], influenza virus neuraminidase [110], *Spodoptera exigua* multiple nucleopolyhedrovirus F protein, single chain antibody fragments and human endogenous retrovirus envelope protein [111] in insect cells also leads to incorporation of the protein into baculovirus envelope. Among these strategies, display of VSVG or heterologous peptide/protein via the VSVG anchor is the most widely adopted and can tremendously enhance baculovirus transduction in vitro and in vivo.

Serum complement proteins (e.g. C5b-9) inactivate baculovirus, hence constituting a major hurdle in the in vivo use of baculovirus. The inactivation problem has been circumvented by the use of complement inhibitors [112] or by displaying human DAF (decay accelerating factor) via gp64 [113]. The DAF-displaying baculovirus caused lower levels of inflammatory cytokines IL-1β, IL-6, and IL-12p40 in macrophages and mitigated liver inflammation in mice when compared with the control virus. These results demonstrate that DAF display offers protection to the baculoviral vector against complement inactivation and attenuates complement-mediated inflammation injury.

4.3.2. Surface modification via capsid display, chemical coupling or electrostatic interactions

Other than the display on the envelope, heterologous protein has been displayed on the capsid by fusion with the major capsid protein VP39. The VP39 fusion with enhanced green fluorescent protein (eGFP) neither interferes with the virus assembly nor affects the virus titer, thereby enabling intracellular baculovirus trafficking and biodistribution monitoring [114]. Similarly, the ZnO binding peptide has been fused to the N-terminus of VP39 while retaining the viral infectivity and conferring the ability to bind nanosized ZnO powders [115]. Besides, by fusing the protein transduction domain (PTD) of human immunodeficiency virus (HIV) TAT protein (a protein responsible for nuclear import of HIV genome) with VP39, the engineered baculovirus results in improved transduction of various mammalian cells

Baculovirus can also be chemically conjugated with compounds such as polyethylene glycol (PEG) alone and folate [116] to improve the transduction of folate receptor-positive KB cells. Additionally, baculoviral vectors have been coated with positively charged polyethylenimine (25 kDa) through electrostatic interactions. The modification imparts baculoviral vectors resistance to human and rat serum-mediated inactivation in vitro and elevates in vivo transduction in the liver and spleen after tail vein injection into mice.

5. Baculovirus display strategies

5.1. Introduction

Recently, a novel molecular biology tool was established by the development of baculovirus surface display [117-123], using different strategies for presentation of foreign peptides and proteins on the surface of budded virions. This eukaryotic display system enables presentation of large complex proteins on the surface of baculovirus particles and has thereby become a versatile system in molecular biology.

The baculovirus system offers great potential as an eukaryotic surface display system, since the post-translational modification of the recombinant proteins is efficient and high transfection rates can be reached. These features are important for the generation of efficient surface display libraries. The principal applications of such strategies are ligand screening of surface expression libraries, for example epitope mapping, antigen display for induction of specific antibodies and presentation of proteins that increase binding to mammalian host cells.

Moreover, display strategies play an important role, as they may be used to enhance the efficiency and specificity of viral binding and entry to mammalian cells. In addition, baculovirus surface display vectors have been engineered to contain mammalian promoter elements designed for gene delivery both in vitro and in vivo. Moreover, baculovirus capsid display has recently been developed; this holds promise for intracellular targeting of the viral capsid and subsequent cytosolic delivery of desired protein moieties. Finally, the viruses can accommodate large insertions of foreign DNA and replicate only in insect cells. Together, these are attributes that are very likely to make them important tools in functional genomics and proteomics.

Display of foreign proteins or peptides on the surface of various virus particles has been valuable in a number of areas within life sciences, ranging from basic research such as protein structure–function studies to diagnostics and gene therapy. One of the most successful examples of display technology is the isolation of antibodies from large combinatorial libraries displayed on the surface of the bacteriophages [124]. The versatile principle of phage display is based on the direct physical linkage between genotype and phenotype. This linkage enables the selection of basically any protein with the desired characteristics, such as increased binding affinity or improved catalytic properties from a suitable display library [125]. Phage display comprises some severe limitations imposed by expression in the bacterial host, however, for example when large complex eukaryotic proteins that require glycosylation or particular protein folding are under study.

Over the past few years, the ability to present large complex glycoproteins on the surface of AcMNPV, has been developed into a versatile system in molecular biology. Expression of proteins or peptides on the baculoviral surface, or more recently also on the viral capsid, without compromising replication in insect cells, has shown to be useful for important applications, both *in vivo* and *in vitro*. The major envelope glycoprotein of AcMNPV is generally known as gp64. The corresponding gene encodes a type I integral membrane glycoprotein with an amino-terminal signal sequence and a carboxy-proximal transmembrane domain. The GP64 protein occurs on the viral particle as a disulphide-linked oligomer, most likely a trimer, and is responsible for viral cell entry mediated by acid-triggered membrane fusion. Structural studies on the GP64 protein have identified separate domains responsible for oligomer formation and membrane fusion. These structural characteristics of gp64 make it a good candidate as a presentation platform for the development of a eukaryotic-based viral surface display system. Modification of viral surface structures by display techniques has enabled the use of baculovirus for enhanced targeting to mammalian cells in vitro. Based on the fact that surface display may interfere with baculovirus infectivity, and that molecules which are displayed on the baculovirus envelope end up in the lysosomes of the mammalian cell and subsequent acid-induced fusion of the viral envelope in the endosomes, an approach for display of foreign protein moieties on the capsid of AcMNPV was recently developed. This system allows for presentation of desired proteins as fusions with the baculovirus major capsid protein VP39. By contrast, molecules displayed on the baculovirus capsid should escape endosomes and thereby follow the capsid through the cytoplasm into the nucleus of trans-

duced mammalian cells. Ideally, capsid display should thus enable transfer of functional molecules into the cytoplasm and/or the nucleus of the target cells.

5.2. Baculoviral display cloning

In the first constructions used to display peptides on the surface of the budded virions the foreign open reading frames were fused to the complete GP64 coding sequence, with the parental baculovirus retaining a wild type GP64 copy [126]. The foreign genes were cloned between the gp64 signal peptide and the mature gp64 peptide. The mechanism of incorporation into the viral particle probably involves the oligomerisation of the fusion construct with wild-type GP64. Until now, only small peptides have been inserted into the protein gp64 [127, 128]. When entire proteins were inserted the virus budding efficiency decreased drastically, and titres similar to those of gp64-deletion mutants were obtained [129]. By comparison of different positions within the gp64 sequence using specific antibody epitopes, it was found that the surface probability of the inserted peptide strongly depends on the position, structural framework and the adjacent amino acids [128]. Incorporation of the fusion protein onto the viral surface usually represents only a small proportion of the total fusion protein and the levels of incorporation into the budded virus are variable and cannot be predicted. The position not only affects the viral titres obtained, but also influences the presentation of the epitope. In addition to the oligomerization domain and fusion domain, the N-terminal part of the protein also contains essential structural or sequential motifs that are more sensitive to changes than the rest of the protein.

Different promoters for the GP64 fusion protein have been evaluated to increase incorporation rates and presentation of the displayed peptide [130]. It was noticed that the use of early promoters resulted in more complete post-translational processing of glycoproteins; but the level of fusion protein detected on the surface of cells and budded virus particles was significantly enhanced when strong, very late polyhedrin promoter was used. High concentrations of the target protein are required on the cell surface in order to reach a signal-to-noise ratio that allows cell sorting to be performed by fluorescence-activated cell sorting, which, at the moment, is the only practical technique for selecting specific clones from baculovirus surface display libraries.

As an alternative to using either the entire GP64 or portions of GP64 protein as the scaffold for protein presentation, the coat protein of a different virus, vesicular stomatitis virus (VSV), or its membrane anchor domain, has also been evaluated. It was shown that by using this strategy, incorporation eGFP was extremely high [131]. The avidity of the display virus increased significantly, without putting a direct limit on the size of the target gene. In the latter cases, wild-type gp64 was still expressed in order to maintain efficient infectivity.

6. Conclusions and perspectives

The study of baculoviruses is a traditional field in virology. In particular, genetic engineering of AcMNPV emerged in the 1980s, and several systems for various purposes have been

developed. However, although genetic engineering of baculoviruses seems to be a thoroughly explored area, much work is still required to fully exploit the advantages of the system.

Vectors and cells with many advantageous characteristics have been developed; yet, it would be tantalizing to assemble all these features in a single system. As mentioned before, the addition of an IRES to the transfer vector to couple the recombinant ORF to an essential BV ORF enhanced the genetic stability of the recombinant virus providing sustained recombinant protein expression. However, this feature is still not commercially available and is not compatible with many commercial systems. Other alternatives that have been explored and may be assembled in new generation systems include: selection by rescue of a lethal gene deletion, deletion of baculovirus chitinase and cathepsin, expression of chaperones and other folding proteins and expression of mammalian glycosylation pathway proteins.

The use of transgenic cell lines for expression of recombinant proteins is a convenient alternative to baculovirus infection, since the protein is not expressed in an infection context. Selection systems for the generation of transgenic insect cell lines may be optimized. Systems based on site-specific recombination would increase the rate of transgenic cell generation, thus simplifying clonal cell isolation. Negative selection systems should be explored as well. Additionally, the development of inducible expression systems would be very convenient, since they are convenient for expression of proteins that affect cell physiology.

The improvement of baculovirus bioinsecticides by means of genetic engineering is a challenging subject. Genetic stability of recombinant baculovirus is an issue, and it is addressed by strategies such as the addition of an IRES (as mentioned before) and by deleting small regions with high recombination rates. Many genes from various sources are being tested for their ability to increase baculovirus biopesticidal propierties, although small RNA-mediated silencing will probably emerge as an important alternative to foreign gene expression approach. Host range modification is even more challenging. To address this question a systematic study could start by replacing each of the baculovirus genes with related baculovirus homologs in search of functional complementation. Other various approaches may be envisaged. Also, bioinformatics studies in search of genes subjected to positive pressure are valuable to provide candidates of host-specific interaction genes. These bioinformatics studies should be updated with the recently sequenced baculovirus genomes. The use of baculovirus to transduce mammalian cell lines and mammalian organism bring baculovirus in the gene therapy and vaccine fields. One of the most challenging objectives in this area is the programming of viral particles to target specific tissues or cell types. In this direction, the replacement of the baculovirus fusion protein by other fusion proteins have shown to modify baculovirus BV tropism, and the development of targeted baculovirus is crucial for exploiting their potential as gene therapy vectors.

From this overview of the field, it is clear that there is room for many strategies and approaches to improve the various applications of genetically engineered baculoviruses.

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