Abstract:
The present invention relates to methods for the production of biopharmaceuticals implementing a baculovirus-based system. These methods advantageously allow the production of biopharmaceuticals with a reduced number of or without contaminating baculoviral virions.
The present invention relates to methods for the production of biopharmaceuticals implementing a baculovirus-based system. These methods advantageously allow the production of biopharmaceuticals with reduced or no contaminating baculoviral virions.

Over the past two decades the baculovirus-insect cell technology has become a very frequently used eukaryotic expression system for the production of recombinant proteins, not only for scientific purposes, but more and more for human and veterinary medicine (Condreay and Kost, 2007, van Oers, 2006). In particular, recombinant baculoviruses derived from Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) are widely employed for large-scale production of heterologous proteins in cultured insect cells. The main reasons for the frequent application of this system are: (1) high levels of expression of foreign proteins, (2) insect cells are able to grow in a suspension culture and thus are easy to scale up, (3) the proteins synthesized in insect cells are processed and modified post-translationally, (4) well-developed manipulation techniques for the viral vectors resulting in a flexible expression system, and 5) non-pathogenic to humans, as the baculovirus host range is restricted to insects and invertebrates. Recombinant baculovirus vectors are being used for the production of individual proteins, as for sub-unit vaccine purposes, but also for higher order structures containing one or more proteins, such as enzyme complexes, viruses or virus-like particles.

Virus-like particles (VLPs) are highly organised structures that self-assemble from virus-derived structural proteins. These stable and versatile nano-particles possess excellent adjuvant properties capable of inducing innate and acquired immune responses (Ludwig & Wagner, 2007). During the past years, VLPs have been applied in other branches of biotechnology taking advantage of their structural stability and tolerance towards manipulation to carry and display
heterologous molecules or serve as building blocks for novel nanomaterials. For immuno-therapeutic and prophylactic applications, many types of virus-like particles (VLP) have been successfully produced in baculovirus-infected insect cells (Noad & Roy, 2003, van Oers et al., 2006, Ramqvist et al., 2007). The first commercial achievement of baculovirus VLP technology for use in humans is the human papillomavirus (HPV) vaccine recently marketed by GlaxoSmithKline, prophylactic against HPV strains 16 and 18. The L1 protein of each of these types of HPV was expressed via a recombinant baculovirus vector and the resulting VLPs were combined to produce the vaccine Cervarix™ (Harper et al., 2006).

Today, there is a huge effort to develop baculovirus-dehved influenza virus-like particles as well as influenza subunit-vaccines as a new generation of non-egg and non-mammalian cell culture-based candidate vaccine. Non-replicating influenza virus-like particles are effective in eliciting a broadened, cross-clade protective immune response to proteins from emerging H5N1 influenza isolates giving rise to a potential pandemic influenza vaccine candidate for humans that can be stockpiled for use in the event of an outbreak of H5N1 influenza (Bright et al., 2008). An influenza subunit vaccine produced in insect cells is close to FDA approval (Cox and Hollister, 2009). Similar strategies could in principle be applied for vaccines against the pandemic influenza such as the recent outbreak of Swine flu.

For gene therapy purposes, baculovirus-insect cell technology is also being applied for the production of infectious adeno-associated virus vectors (e.g. Urabe et al., 2002) and lentiviral vectors (Lesch et al., 2008). For the production of AAV vectors insect cells are co-infected with three recombinant baculoviruses - One producing the AAV replicase (REP) proteins, one carrying the cap functions for producing the AAV viral structural proteins (VP1, VP2, VP3), and a third baculovirus comprising an AAV-ITR vector with the ability to carry and transfer transgenes. Recently an improved version of this production had been published which is based on the use of only recombinant baculoviruses, one of them carrying the rep and cap functions of AAV (Smith et al. 2009). The produced AAV vector is indistinguishable from that produced in mammalian cells in its physical and
biological properties. The yield of the AAV-ITR vector particles approached $5 \times 10^4$ per Sf9 insect cell demonstrating that the system is able to produce high quantities of AAV vectors in a simple manner. Currently, clinical trials with baculovirus-derived AAV vectors are underway for instance for lipoprotein lipase deficiency (Amsterdam Molecular Therapeutics B.V.). As an alternative, scalable approach to produce lentiviral vectors (Lesch et al., 2008) mammalian 293T cells were transduced simultaneously by four recombinant baculoviruses produced in insect cells to express all elements required for generation of a safe lentivirus vector. The unconcentrated lentiviral titers in mammalian cell culture media were on average $2.5 \times 10^6$ TU ml$^{-1}$, comparable to titers of the lentiviruses produced by conventional four-plasmid transfection methods. In addition, there is a general effort to convert lentiviral vector production methods into better scalable insect cell-based technologies.

Tjia et al., 1983 discovered that BVs can be internalized by mammalian cells and even some of the viral DNA reached the cell nucleus. Further studies showed that baculoviruses can enter mammalian cells and mediate expression of *Escherichia coli* chloramphenicol acetyl-transferase under the Rous sarcoma virus promoter (Carbonell et al., 1985). These findings led to the development of novel baculovirus-based gene delivery vehicles for mammalian cells (Boyce & Bucher, 1996, Hofmann et al., 1995, Condreay and Kost, 2007, Kaikkonen et al., 2008). Today, there is strong evidence that baculovirus-dehved gene delivery vectors can mediate transient and stable expression of foreign genes in mammalian cells following antibiotic selection (Lackner et al., 2008).

There is still poor knowledge about transcriptional activities of baculovirus promoters in mammalian cells. It has been demonstrated that the transactivator protein IE1 of AcMNPV is functional in mammalian cells (Murges et al., 1997) as well as the early-to-late (ETL) promoter (Liu et al., 2006a,b). Among the other imperfectly explored areas is the interaction of baculoviruses with components of the mammalian immune system. AcMNPV virus is able to induce antiviral cytokine production, which protects cells from infection with vesicular stomatitis virus and influenza virus (Abe et al., 2003, Gronowski et al., 1999). AcMNPV is also
recognized by Toll-like receptor 9 on dendritic cells and macrophages, and AcMNPV induces antitumor acquired immunity (Kitajima & Takaku, 2008). These results suggest that AcMNPV has the potential to be an efficient virus or tumor therapy agent which induces innate and acquired immunity. In spite of universally positive effects of AcMNPV on components of the humoral and adaptive cell-mediated immunity in mice, the interaction of baculoviruses with the human immune system can be slightly different. Additionally, immunoadjuvant properties of AcMNPV should be fully separated from immune response against target vaccine/biopharmaceuticals produced in insect cells.

These features of baculoviruses are strongly disadvantageous in cases where baculoviruses are utilized for the production of vaccines or viral vectors for therapeutical purposes (e.g. AAV, lentivirus). Contamination of the produced biopharmaceuticals with both types of baculovirus virions - budded virions (BVs) and occlusion-derived virions (ODVs) should, therefore, be avoided. In general, the recombinant proteins can be produced in insect cells as cytosolic, membrane-bound, or extra-cellularly secreted proteins. The latter secreted proteins are highly "contaminated" with baculoviral BVs present in the culture medium. It can be very difficult to separate undesirable baculovirus virions from produced recombinant biopharmaceuticals in some production and purification configurations. It has been shown for instance that these BVs can cause problems during the purification process of AAV vectors produced with baculovirus-insect cell technology (personal communication O. Merten, Genethon). On the other hand, there are also ODVs, always formed inside the nuclei of infected cells, in all conventional baculovirus-insect cell expression systems, even if occlusion bodies are not formed, due to replacement of the polyhedrin open reading frame by a desired gene. Analogously, these virions can co-purify with intracellular produced recombinant proteins or VLPs during purification process.

In summary, the separation of recombinant proteins and, especially, VLPs from baculovirus particles, requires a lot of effort and occurs at high costs. In addition, it results in reduced efficiency of recombinant protein production. Therefore, the development of an improved baculovirus-insect cell technology allowing high
expression of heterologous proteins while eliminating baculovirus BV and ODV production is highly desirable, and is the topic of this patent application. Such a baculovirus vihon-free production system would represent a significant improvement over existing systems for the production of all kinds of biopharmaceuticals in insect cells.

The present invention is based on the identification of efficient baculovirus-insect cell based methods for producing biopharmaceuticals with reduced amounts or absence of baculovirus virions.

An object of the present invention thus provides a method for the production of a biopharmaceutical product, comprising:

(a) infecting a biopharmaceutical-producing insect cell with at least one baculovirus, said at least one baculovirus comprising a genome coding for said biopharmaceutical product, and

(b) maintaining the biopharmaceutical-producing insect cell under conditions such that the biopharmaceutical product is produced, wherein each genome of said at least one baculovirus is deficient for at least one gene essential for proper baculovirus virion assembly or wherein said biopharmaceutical-producing insect cell comprises an expression control system allowing the inactivation of at least one gene essential for proper baculovirus virion assembly.

In an embodiment, the invention relates to the above method, wherein said at least one gene essential for proper baculovirus virion assembly is made deficient in said genome by mutation, for example by way of nucleotide substitution, insertion or deletion.

In another embodiment, the invention relates to the above method, wherein the biopharmaceutical-producing insect cell is a recombinant insect cell comprising a construct expressing a dsRNA specific for the at least one gene essential for proper baculovirus virion assembly, the dsRNA being optionally expressed under the control of an inducible promoter.
In a further embodiment, the invention relates to the above method, wherein the at least one baculovirus is produced before step (a) in a baculovirus-producing cell expressing a complementing copy of the at least one gene essential for proper baculovirus virion assembly.

In yet another embodiment, the invention relates to the above method, wherein the at least one gene essential for proper baculovirus virion assembly is selected from vp80, vp39, vp1054 and p6.9.

In another embodiment, the invention relates to the above method, wherein the deficiency or inactivation of the at least one gene essential for proper baculovirus virion assembly does not affect very late gene expression from said baculovirus in comparison to very late gene expression from the wild-type baculovirus vector.

In yet another embodiment, the invention relates to the above method, wherein the at least one baculovirus is preferably derived from AcMNPV or Bombyx mori (Bm) NPV.

In a further embodiment, the invention relates to the above method, wherein the biopharmaceutical product is a recombinant protein, a recombinant virus, a virus-derived vector, or a virus-like particle.

In another embodiment, the invention relates to the above method, wherein the biopharmaceutical product is a recombinant AAV vector. Furthermore, the invention relates to the above method, wherein the biopharmaceutical product is a vaccine. Representative examples of vaccines than can be produced with the method of the present invention include, but are not limited to, influenza virus-like particles or influenza subunit vaccines, and vaccines against Human papillomavirus.

In a further embodiment, the invention relates to the above method, wherein the biopharmaceutical product is coded by at least one gene introduced in the
recombinant baculovirus genome under the control of a baculovirus promoter, preferably the p10 or polyhedrin promoter.

Another object of the invention provides the use of a baculovirus-insect cell system for the production of a biopharmaceutical product wherein the baculovirus-insect cell system comprises a biopharmaceutical-producing insect cell infected with at least one recombinant baculovirus, wherein:
- the, or each, recombinant baculovirus comprises a recombinant baculovirus genome that encodes the biopharmaceutical product, or at least one component of the biopharmaceutical product, and
- the recombinant baculovirus genome is deficient for at least one gene essential for proper assembly of said baculovirus, or the biopharmaceutical-producing insect cell comprises an expression control system allowing the inactivation of the at least one gene essential for proper baculovirus virion assembly.

Yet another object of the invention relates to a bacmid comprising a baculovirus genome, wherein said genome is deficient for a gene essential for proper baculovirus virion assembly, preferably wherein the genome of said baculovirus is deficient for vp80, vp39, p6.9 or vp1054. In a particular aspect, said bacmid is derived from AcMNPV and is lacking the vp80 ORF.

A further object of the invention relates to a recombinant AcMNPV baculovirus vector, wherein the genome of said baculovirus is deficient for a gene essential for proper baculovirus virion assembly, preferably wherein the genome of said baculovirus is deficient for vp80, vp39, vp1054 or p6.9. In a particular aspect, the invention relates to a recombinant AcMNPV baculovirus lacking the vp80 ORF.

The invention has also as an object an insect cell infected with the above mentioned recombinant AcMNPV baculovirus.

Another object of the invention relates to an insect cell, comprising a construct expressing a dsRNA specific for a gene essential for proper baculovirus virion
assembly, preferably directed against \( \text{vp80, vp39, vp1054} \) and/or \( \text{p6.9} \), said construct being preferably integrated in the genome of the insect cell.

A further object of the invention relates to an insect cell comprising an expression cassette coding for a gene essential for proper baculovirus virion assembly. In particular, the invention relates to said insect cell, wherein the gene coded by the expression cassette is \( \text{vp80, vp39, vp1054} \) and/or \( \text{p6.9} \).

Another object of the invention relates to a method for the production of a baculovirus deficient for at least one gene essential for proper baculovirus virion assembly, comprising the step of transfecting an insect cell comprising an expression cassette coding for a gene essential for proper baculovirus virion assembly, with a bacmid comprising a baculoviral genome, wherein said genome is deficient for a gene essential for proper baculovirus virion assembly, preferably wherein the genome of said baculovirus is deficient for \( \text{vp80, vp39, p6.9} \) and/or \( \text{vp1054} \), wherein the gene essential for proper baculovirus virion assembly deficient in said bacmid is the gene coded by the expression cassette comprised in said insect cell.

The present invention relates to the production of biopharmaceuticals in insect cells by implementing a baculoviral system, but without coproduction of contaminating baculovirus virions. The methods of the invention simplify the downstream processing of biopharmaceuticals produced in insect cells to a large extent.

Thus, the invention relates to methods for the production of a biopharmaceutical product implementing a baculoviral system designed to avoid the production of contaminating baculoviral virions. The method of the present invention comprises the infection of biopharmaceutical-producing insect cells with at least one baculovirus coding for said biopharmaceutical product.

Baculoviruses are enveloped DNA viruses of arthropods, two members of which are well known expression vectors for producing recombinant proteins in cell
cultures. Baculoviruses have circular double-stranded genomes (80-200 kbp) which can be engineered to allow the delivery of large genomic content to specific cells. The viruses used as a vector are generally *Autographa californica* multcapsid nucleopolyhedrovirus (AcMNPV) or *Bombyx mori* (Bm)NPV (Kato et al., 2010).

Baculoviruses are commonly used for the infection of insect cells for the expression of recombinant proteins. In particular, expression of heterologous genes in insects can be accomplished as described in for instance U.S. 4,745,051; Friesen et al. (1986); EP 127,839; EP 155,476; Vlak et al. (1988); Miller et al. (1988); Carbonell et al. (1988); Maeda et al. (1985); Lebacq-Verheyden et al. (1988); Smith et al. (1985); Miyajima et al. (1987); and Martin et al. (1988). Numerous baculovirus strains and variants and corresponding permissive insect host cells that can be used for protein production are described in Luckow et al. (1988), Miller et al. (1986); Maeda et al. (1985) and McKenna (1989).

According to the present invention, any genome derived from a baculovirus commonly used for the recombinant expression of proteins and biopharmaceuticalal products may be used. For example, the baculovirus genome may be derived from for instance AcMNPV, BmNPV, *Helicoverpa armigera* (HearNPV) or *Spodoptera exigua* MNPV, preferably from AcMNPV or BmNPV. In particular, the baculovirus genome may be derived from the AcMNPV clone C6 (genomic sequence: Genbank accession no. NC_001623.1 - SEQ ID NO:1).

The terms "Biopharmaceutical", "Biopharmaceuticals" and "Biopharmaceutical Product" are intended to define medical drugs produced using biotechnology. As such, biopharmaceuticals may correspond to recombinantly produced drugs such as recombinant proteins, notably recombinant hormones or recombinant proteins for use as vaccines, viruses, for example therapeutic recombinant AAV or other viral vectors for use in gene therapy, as well as virus-like-particles (or VLPs). Such biopharmaceuticals are intended to be administered to a subject in need thereof for the prophylactic or curative treatment of a disease condition in said subject which may be of either human or animal origin.
A biopharmaceutical product may correspond to a single chain protein or peptide, for example in the case of a therapeutic recombinant protein, or may be a complex structure such as a virus or a virus-like particle. In the latter two cases, the components of the complex may be expressed from several recombinant baculoviruses, each carrying at least one component of the complex structure, or from a single baculovirus whose genome has been genetically modified by the insertion of sequences encoding all the components of the complex. For example, for the production of a recombinant AAV, a system comprising three baculoviruses may be used: a baculovirus coding for the AAV Rep proteins, a baculovirus coding the AAV Cap proteins and a baculovirus coding the AAV-ITR genome comprising a therapeutic gene between the two AAV ITRs. A system comprising two baculoviruses is also available now, for which the DNA sequences coding for the AAV Rep proteins and the AAV Cap proteins are provided by one baculovirus.

In a preferred embodiment of the invention, the heterologous gene(s) encoding the biopharmaceuticals are placed under the control of a baculoviral promoter. For example, the heterologous gene(s) is (are) placed under the control of the polyhedrin or p10 promoter, or of any other baculoviral promoter commonly used for expression in an insect cell (e.g. ie-1, p6.9, gp64 or the Orchylia pseudotsugata (Op) MNPV ie-2 promoter). In a preferred embodiment of the invention, the baculoviral promoter is selected from very late expression promoters, for example from the p10 and polyhedrin promoters, preferably under the control of the polyhedrin promoter.

In the method of the present invention, at least one gene essential for proper baculovirus virion assembly is either absent from the genome of the recombinant baculovirus(es) implemented in the above described method, or its expression is prevented. The inventors have shown that the deletion or inactivation of such genes results in the reduction, or even the complete absence, of budded virions and/or occlusion derived virions, the two forms of a baculovirus.
A "gene essential for proper baculovirus virion assembly" is a gene whose deficiency or inactivation in a baculovirus-producing cell negatively impacts the number of BVs and ODVs produced from said cell. Such a gene may be identified as provided in the herein below examples. In particular, one can use double stranded RNAs specific for a particular baculoviral gene to assess the impact of the absence of said particular gene on the production of BVs and ODVs, for example by detecting the expression of a reporter gene present in the baculoviral genome in the cell culture, and thus determine the spreading or absence of spreading of the baculovirus (single-infection phenotype). Alternatively baculovirus virions may be detected by the presence of baculoviral structural proteins or genome sequences in the culture medium when sampling for BV production. Both virion types may be detected by electron microscopy.

In a preferred embodiment of the invention, the gene essential for proper baculovirus virion assembly is selected from \textit{vp80}, \textit{vp39}, \textit{vp1054} and \textit{p6.9}. More preferably, the gene is selected from \textit{vp80} and \textit{vp39}, said gene being preferably \textit{vp80}.

The invention provides the inactivation of genes essential for proper baculovirus virion assembly. Several strategies may be implemented for this purpose, and in particular: the mutation, for example by deletion, of the selected gene(s) in the recombinant baculovirus genome; or the reduction of the expression of the selected gene by an expression control system provided in the biopharmaceutical-producing insect cell intended to be infected by the baculovirus. Preferably, the expression control system involves the down-regulation by RNA interference of the expression of the protein(s) encoded by the selected gene(s).

In one embodiment of the invention, the genome of the at least one baculovirus implemented in the method of the invention is deficient for at least one gene essential for proper baculovirus virion assembly, in particular for a gene coding for \textit{vp80}, \textit{vp39}, \textit{vp1054} and/or \textit{p6.9}, preferably for \textit{vp80} and/or \textit{vp39}, and even more preferably for \textit{vp80}. More particularly, said genome is derived from AcMNPV, more particularly from AcMNPV clone C6 genome sequence (Genbank accession no.
NC_001 623.1 - SEQ ID NO: 1). Accordingly, in one aspect the invention provides the method as defined above, wherein the baculoviral genome is an AcMNPV genome, in particular an AcMNPV clone C6 genome, deficient for the gene coding for vp80, vp39, vp1054 and/or p6.9, preferably for vp80 and/or vp39, and even more preferably for vp80. As is well known in the art and specified in Genbank accession no. NC_001 623.1, these genes are positioned as follows in AcMNPV clone C6 genome (i.e. in SEQ ID NO:1): positions 89564-91 639 for vp80; positions 75534-76577 for vp39 (complementary sequence); positions 45222-46319 for vp1054; positions 867 1 2-86879 for p6.9 (complementary sequence).

It should be noted that in case the biopharmaceutical product is a complex product comprising various subunits each encoded by different baculoviruses, the genomes of all the implemented recombinant baculoviruses are deficient for the selected essential gene, so as to avoid complementation of one genome by another. In other words, when several baculoviruses are used to infect the same biopharmaceutical-producing insect cell, each of these baculoviruses are deficient for the same gene(s) essential for proper baculovirus virion assembly.

According to the present invention, a gene may be made deficient by mutating said gene. A mutation of a gene essential for proper baculovirus virion assembly is a modification of said gene that results in the complete absence of a functional essential gene product. Accordingly, said mutation may result in the introduction of one or several stop codon in the open reading frame of the mRNA transcribed from the gene essential for proper baculoviral virion assembly or may correspond to the deletion, either total or partial, of the gene essential for proper baculovirus virion assembly. A gene essential for the proper baculoviral virion assembly may be mutated by way of nucleotide substitution, insertion or deletion in the sequence of all or a part of the wild type gene (for example in the sequence provided in Genbank Accession No. NC_001 623.1, for a genome derived from AcMNPV). The mutation may correspond to the complete deletion of the gene, or to only a part of said gene. For example, one may delete at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80% and even
preferably more at least 90% of the gene essential for proper baculoviral virion assembly.

The mutant baculoviral genome may be produced using standard methods well known in the art, such as site-directed mutagenesis (see, e.g., Sambrook et al. (1989)) and Lambda red recombination (Datsenko & Wanner, 2000). The gene essential for proper baculovirus virion assembly may in particular be deleted as provided in the below examples. In summary, one can make use of the mutant loxP sites described by Suzuki at al. (2005), by replacing either totally or in part the gene essential for proper baculovirus virion assembly with a reporter gene flanked by mutant LoxP sites by recombination. The reporter gene (for example the gene coding for chloramphenicol acetyl transferase (cat) is then excised by implementing a recombination with Cre recombinase.

This embodiment is illustrated in the below examples and is detailed for baculoviruses whose genome has been modified by deleting a 2074-bp fragment of the vp80 ORF in the AcMNPV genome. This particular genome is part of the present invention, but is given as a non limiting example of what is a mutant baculoviral genome according to the invention.

It should be noted that recombinant engineering of the baculovirus genome may result in the insertion of several sequences like cloning sites or recombination sites (for example one remaining LoxP site after recombination with Cre recombinase). This is irrelevant as long as the resulting genome is made deficient for the selected gene essential for proper baculovirus virion assembly.

In this embodiment, wherein the genome of the at least one baculovirus is deficient for at least one gene essential for proper assembly of baculovirus virion, the production of recombinant budded baculovirus particles needed for the initial infection of the cells producing the bio-pharmaceuticals requires the implementation of special cells rescuing the deficient gene, i.e. these baculovirus-producing cells express the selected gene. In other terms, the baculovirus-producing cell expresses a complementing copy of the at least one gene essential for proper baculoviral virion assembly which is deficient in the baculovirus
genome. For example, a Sf9-derived cell line constitutively producing the product of the gene essential for proper assembly of the baculovirus virion may be established. This recombinant cell line is used for production of baculovirus seed stock while conventional insect cell lines like Sf9, Sf21 or High-five cell lines can be infected with the produced baculovirus for heterologous expression of the biopharmaceutical product. Accordingly, the invention also relates to an insect cell modified so as to express a gene essential for proper baculovirus assembly, said gene being mutated in a baculovirus used for the production of biopharmaceuticals, as defined above. Such a cell line used for the production of the mutant baculovirus vector implemented in the method of the present invention is referred to as a "baculovirus-producing cell". When the baculovirus genome is deficient for a gene essential for proper baculovirus virion assembly, the baculovirus-producing insect cell must provide and express said gene in order to complement the deficiency and to produce an infectious baculovirus. In a particular embodiment, the insect cell used for the production of the baculovirus is modified by transfection with an expression cassette coding for at least one gene essential for proper baculovirus virion assembly. In an embodiment, said expression cassette is integrated in the genome of said cell. One may also use insect cells transiently transfected with at least one plasmid comprising the expression cassette. The terms "expression cassette" denote a construct comprising the coding sequence of a gene of interest functionally linked to expression control sequences. Such an expression cassette may be a plasmid comprising the ORF of a gene essential for proper baculovirus virion assembly placed under the control of a promoter functional in the selected insect cell, and does not contain baculoviral genome sequences other than the gene essential for proper baculovirus virion assembly to be complemented and optionally the promoter sequence allowing the expression of said gene (in particular, an expression cassette is not a bacmid or any other baculoviral entire genome). Exemplary expression control sequences may be chosen among promoters, enhancers, insulators, etc. In one embodiment, the complementing gene is derived from the genome of the baculovirus in which the gene essential for proper baculovirus virion assembly has been made deficient. In another embodiment, the complementing gene originates from the genome of a different baculovirus species.
than the baculovirus genome used for the production of biopharmaceuticals. For example, the baculovirus used for the production of biopharmaceuticals may be derived from the AcMNPV genome, and the complementing gene introduced in the baculovirus-producing cell is derived from BmNPV or SeMNPV. More specifically, the baculovirus genome may be made deficient for vp80, vp39, vp1054 and/or p6.9 and the baculovirus-producing cell may comprise a copy of a gene from BmNPV or SeMNPV able to complement these genes (e.g. as provided in the examples, p6.9 is deleted in the AcMNPV genome and the baculovirus-producing cell provides a rescuing copy of SeMNPV p6.9 gene).

The invention thus also provides a method for the production of a baculovirus deficient for at least one gene essential for proper baculovirus virion assembly, comprising the step of transfecting an insect cell comprising an expression cassette coding for a gene essential for proper baculovirus virion assembly, with a bacmid comprising a baculoviral genome, wherein said genome is deficient for a gene essential for proper baculovirus virion assembly, preferably wherein the genome of said baculovirus is deficient for vp80 or vp39, p6.9 and/or vp1054, wherein the gene essential for proper baculovirus virion assembly deficient in said bacmid is the gene coded by the expression cassette comprised in said insect cell. According to this method, the gene deficient in the baculoviral genome is complemented by the gene expressed in the insect cell. The cells transfected with the bacmid are maintained in conditions such that baculovirus virions are produced. These produced baculovirus virions, which comprise a genome where at least one gene essential for proper baculovirus virion assembly is lacking, are then collected for their subsequent use for infecting biopharmaceutical-producing insect cells for the production of the biopharmaceutical.

In the embodiment where the genome of the baculovirus is deficient for at least one gene essential for baculovirus virion assembly, the biopharmaceutical-producing insect cell must be unable to complement the deficiency of said gene. Otherwise, the deficiency would be rescued by the biopharmaceutical-producing cell and BVs and ODVs might be produced. The presence or absence of a gene essential for proper baculovirus assembly may be monitored for example by
checking said cell by a PCR specific to said gene or by detection of the protein product of this gene (for example by western-blot with an antibody specific to said gene product). Cells expressing a functional product of the gene essential for proper baculovirus virion assembly which has been made deficient in the genome of the implemented baculovirus intended to infect said cell must be disregarded as bio-pharmaceutical producing cells.

In another embodiment of the invention, the expression of the gene essential for proper assembly of baculovirus virions is controlled by an expression control system. The term "expression control system" defines a modification of the baculovirus-producing insect cell system/ the biopharmaceutical-producing cell system and/or yet another adaptation of the viral genome, resulting in the specific regulation of the gene essential for proper baculovirus virion assembly. This system may be an inducible expression system (for example Tet-On, Tet-Off, ecdysone-based systems (Dai et al., 2005) or baculovirus homologous region (hr) containing elements, such as the hr2 system described by Aslanidi et al., (2009) allowing the desired triggering or shutdown of the essential gene, an RNA interference expressing construct or a combination of these.

In a particular embodiment, the expression of the gene essential for proper assembly of baculovirus is inactivated by RNA mediated silencing, or RNA interference (Salem & Maruniak, 2007, Kanginakudru et al., 2007). Preferably, a insect-cell derived cell line, in particular a Sf9-dehved cell line, is established by stably transforming such a cell with a construct coding for a gene-specific double stranded RNA (dsRNA) to silence the expression of the gene essential for proper baculovirus virion assembly. This dsRNA expressing cell line is used for the expression of the biopharmaceutical product after infection with the recombinant baculovirus(es) carrying the gene coding for said biopharmaceutical product. In this embodiment, seed stock recombinant baculovirus(es) may be produced with conventional Sf9, Sf21 or High-Five cell lines (i.e. without the need of a complementing copy of the gene in the cell), since in this case the baculovirus genome comprises the wild-type gene essential for proper baculovirus virion assembly.
In yet another embodiment of the invention, the gene essential for proper baculovirus virion assembly is placed under the control of an inducible promoter, allowing either the expression or repression of said gene under controlled conditions.

In a preferred embodiment, the number of baculovirus virions produced in the method of the present invention is reduced by at least 50% in comparison to the number of baculovirus otherwise produced by the biopharmaceutical-producing cell using a baculovirus genome comprising all the genes essential for proper baculovirus virion assembly. More preferably, the number of baculovirus virions is reduced by at least 60%, at least 70%, at least 80%, at least 90% and most preferably by at least 95% in comparison to a wild type baculovirus genome.

As discussed above, the use of insect cell/baculovirus systems for the production of biopharmaceuticals in the prior art is characterized by the co-production of huge quantities of recombinant baculoviruses (and may be over $10^8$ pfu/ml) in parallel to the biopharmaceutical product, needing carefully developed and optimized downstream processing protocols to inactivate and eliminate this baculovirus contamination. Inactivation can be performed by the addition of a detergent step leading to disintegration of the lipid layer of the contaminating baculovirus, such as used for the purification of virus like particles for vaccine purposes (porcine parvovirus-VLPs (Maranga et al. (2002)) or rotavirus-VLPs (Mellado et al. (2008)) or the purification of different serotypes of AAV (Smith et al. 2009).

Further efficient separation steps have been used: centrifugation (Wang et al. (2000); Maranga et al. (2002); Mellado et al. (2008)), microfiltration (Tellez. (2005)) negative elimination of baculovirus proteins (e.g. Mellado et al. (2008)) or positive affinity chromatography (retention/capture of a biopharmaceutical - flow through of the contaminating proteins, such as capture of the vp7 protein of rotavirus by Concanavalin A chromatography (Mellado et al. (2008)), capture of the immunogenic chimeric rVP2H infectious bursal disease virus particles by immobilized metal-ion affinity chromatography (Wang et al. (2000)) or capture of different AAV serotypes by immunoaffinity chromatography using cameld
antibodies (Smith et al. 2009). In particular, due to the use of highly specific
immunoligands, the use of immunoaffinity allows the complete separation of the to
be purified biopharmaceutical (e.g. specific AAV) from any contaminant, and in the
case of the biopharmaceutical (e.g. specific AAV) from any contaminant, and in the
case of the baculovirus system, from the huge contamination by baculovirus due
to the concomitant production of baculovirus in parallel to the biopharmaceutical.

These references present very clearly the need of these different process steps for
inactivating and eliminating residual baculovirus contaminants, because without
these steps, the biopharmaceutical product is still considerably contaminated by
various baculovirus proteins and cannot be used for clinical purposes.

The method of the present invention allows a significant reduction of the number of
contaminating baculovirus virions, or even a complete absence. As a
consequence, a reduced number of purification steps will be necessary for getting
a biopharmaceutical for clinical purposes (or even no purification step if no
baculoviral virion is produced). Thus, the biopharmaceutical production and
purification protocol is simplified because by using the method of the present
invention, the need for eliminating residual baculovirus virion is greatly reduced. In
case a simplified purification protocol is still to be applied, the skilled artisan may
select at least one of the above identified methods and protocols to obtain a
purified biopharmaceutical product.

Preferably, the selected essential gene is a gene whose inactivation does not
affect baculoviral very late gene expression, compared to the original baculovirus
vector. In the AcMNPV genome (and other alpha-baculoviruses), the p10 and
polyhedrin promoters are the very late expression promoters and it should be
noted that in baculovirus/insect cell production systems, the heterologous gene is
most commonly inserted under the control of these very strong promoters allowing
expression of very large amounts of recombinant proteins. The inactivation of a
gene essential for proper baculovirus virion assembly, which does not affect very
late gene expression is thus preferred. The terms "does not affect very late gene
expression" denotes the fact that the level of recombinant protein expression from
very late baculovirus promoter comprised in the genome of a baculovirus modified
according to the invention is at least 70% in comparison to the levels obtained from a non-modified genome, more preferably greater than 80%, more preferably greater than 90%. It should be mentioned that the level of expression of a biopharmaceutical product from a very late baculoviral promoter may even be greater than 100% of the level obtained with the non-modified vector in the method of the present invention.

Among the genes tested by the inventors, the vp80 gene is particularly preferred since its deletion does not affect very late expression, while it totally prevents production of BVs and results in a significant reduction in the number of intracellular nucleocapsids, the precursors of ODVs.

Very late expression may be evaluated by placing a reporter gene, for example a gene coding for a GFP, in particular egfp, or a luciferase gene, under the control of the polyhedrin or p10 promoter in a wild type AcMNPV vector and in a mutant AcMNPV genome from which the essential gene has been inactivated, and by comparing the expression of the product of the reporter gene from both genomes. Preferably, very late expression from the vector with a mutated baculovirus backbone is at least 60% of the expression level obtained with the wild type AcMNPV vector and preferably higher than 80%, more preferably higher than 90%, as measured from a reporter gene under the control of either p10 or polyhedrin gene promoters.

The invention also relates to a method for screening baculoviral genes, the inactivation of which could be useful for producing biopharmaceuticals without contaminating baculovirus virions in an insect cell - baculovirus system as defined above, comprising:

a) providing a cell culture of cells containing a baculoviral genome;

b) contacting said cell culture with means for inactivating at least one test baculoviral gene of said baculoviral genome, for example with RNA interference; and

c) testing virion formation from said cell culture in comparison to virion formation from a cell culture not contacted with said means;
wherein a test gene is selected as potentially useful for producing biopharmaceuticals if its inactivation results in a reduction of baculoviral virion formation.

In a particular embodiment, the method for screening of the invention further comprise step d) of testing very late gene expression from the cell culture contacted with said means in comparison to very late gene expression from a cell culture not contacted with said means;

wherein a test gene is selected as potentially useful for producing biopharmaceuticals if its inactivation results in a reduction of baculoviral virion formation and if it does not affect very late gene expression from said baculoviral genome.

The invention also relates to a method for screening baculoviral genes, the inactivation of which could be useful for producing biopharmaceuticals without contaminating baculovirus virions in an insect cell - baculovirus system as defined above, comprising:

- inactivating at least one test gene of a baculoviral genome (for example by deletion of said test gene in said genome);
- evaluating baculoviral very late gene expression from said baculoviral genome as defined above;
- determining production of baculoviral virions from cells containing said baculoviral genome;

wherein a gene is selected as potentially useful for the production of biopharmaceuticals if its inactivation

- results in a reduction in the production of baculoviral virions, and
- does not affect very late gene expression from said baculoviral genome, as defined above.

In a particular embodiment of the method for screening a baculoviral gene, the inactivation of which could be useful for producing biopharmaceuticals, the inactivation of the test gene is carried out with dsRNA specific for said test gene.
In particular, the candidate baculovirus gene can be identified by knocking down its expression by RNA interference to test its role in virion formation.

The invention will now be illustrated with the following examples, which are provided as non-limiting exemplary embodiments of the invention.

Legends to the figures

Figure 1. dsRNA-mediated gene silencing screening. Insect Sf9 cells were seeded in 24-well tissue culture plates (2^2 \times 10^5 cells/well) in 1 ml Sf-900 II SFM culture medium at 28°C. After two hours, the culture medium was removed, and the cells were infected with recombinant baculovirus carrying the egfp gene under control of the polyhedrin promoter (AcMNPV-EGFP) under standard conditions. (A) Determination of very late gene expression level using fluorescent microscopy. Cells were infected at MOI=I OTCID_{50} units/cell and transfection with gene-specific dsRNA for vp1 054, vp39, vp80, dbp and ec-27 was performed at 1 h post infection (p.i.). The level of very late gene expression was checked by EGFP-specific fluorescence at 48 h p.i. dsRNAs specific for egfp and cat sequences were used as RNAi controls. (B) Measurement of very late gene expression levels by an immunoblotting-based assay. The cells were infected with AcMNPV-EGFP at MOI=I and transfection with gene-specific dsRNA was also performed at 1 h p.i.. The level of very late gene expression was analyzed by using an rabbit anti-EGFP polyclonal antiserum at 48 h p.i. Anti-vp39 and anti-α-tubulin antibodies were used as internal controls. (C) Titration and detection of produced budded virions in dsRNA-treated cells. Budded virions were harvested at 36 hours p.i., and used either for end-point dilution assays to measure titers of infectious virions, or for PCR-based detection to check the presence of virus particles. (D) Presence of occlusion-derived virions and rod-shaped structures in vp39- and vp80-own-regulated cells. The cells were harvested 36 hours p.i., lysed, and the cell lysates were ultracentrifuged through a cushion of 40% sucrose solution (45,000 rpm for 1
hour, Beckman SW55). Pellets were resuspended in demi-water and analyzed by negative staining electron microscopy. The bars represent 100 nm.

**Figure 2. Construction of the AcMNPV vp80-null bacmid. (A)** Strategy for construction of a vp80-null bacmid containing a complete deletion of the AcMNPV vp80 open-reading frame via homologous recombination in E. coli. At the first step, a 2074-bp fragment encompassing the vp80 ORF was deleted and replaced with a sequence cassette containing the chloramphenicol (cat) resistance gene flanked by modified loxP (LE and RE) sites. Subsequently, the antibiotic resistance gene (cat) was eliminated from the bacmid sequence using the Cre/loxP recombination system. The promoter sequence of the p48 gene and the polyadenylation signal of the he65 gene were remained intact. Oligonucleotide pairs were used in PCR analysis of the wild-type locus and two vp80 knock-out genotypes to confirm the deletion of the vp80 ORF and the correct insertion/deletion of the chloramphenicol resistance gene cassette, as indicated by unilateral arrows. Their names are designated according to nucleotide sequence coordinates. Primers for cat gene cassette amplification are named cat-F and cat-R. (B) PCR-based detection of the presence or absence of sequence modifications in the vp80 locus in the original AcMNPV bacmid (Ac-wt), Ac-i/p80null(+cat), and Ac-i/p80null(-cat) bacmids. The top figure confirms the vp80 gene deletion and the insertion of the cat cassette into the vp80 locus with primer pairs 90292/90889 and cat-F/cat-R. The bottom figure is showing PCR-based verification of the correct recombination processes in the vp80 locus using the 89507/91 7 13 primer pair.

**Figure 3. Viral replication capacity of AcMNPV-vp80 knockout and repaired bacmid constructs using transfection-infection assays (A)** Schematic representation of expression cassettes transposed into the polyhedrin locus. Four repair constructs were made (vp80 driven by its native promoter, vp80 driven by the polyhedrin promoter, N-terminally FLAG-tagged vp80 and C-terminally FLAG-tagged vp80, both expressed from its native promoter). The bacmid genome backbones used for transfection assays are indicated on the left. As positive control of viral replication the wild type AcMNPV (bMON14272) bacmid was used. The Ac-gp64null bacmid was used as negative control representing a prototype
bacmid with a "single-cell infection" phenotype. (B) Time course fluorescence microscopy showing the propagation of the infection in Sf9 cells transfected with indicated bacmid constructs. Progress of viral infection was checked by EGFP detection at indicated times post transfection. At 120 hours p.t., the cell culture supernatants were collected to initiate a secondary infection. (C) Secondary infection assay. EGFP was detected at 72 hours p.i. to signal the progress of infection.

Figure 4. Growth curves of AcMNPV-vp80null repaired bacmid constructs generated from a transfection time-course assays. Sf9 cells were transfected with 5.0 µg of DNA from each repair bacmid. (a) vp80 driven by its native promoter, (b) vp80 driven by the polyhedrin promoter, (c) N-terminally FLAG-tagged vp80, and (d) C-terminally FLAG-tagged vp80, both expressed from the vp80 promoter. Cell culture supernatants were harvested at the indicated time points post-transfection and analysed for the production of infectious budded virus by an TCID_{50} end-point dilution assay. Infectivity was determined by monitoring EGFP expression. The points indicate the averages of titers derived from three independent transfections, and the error bars represent the standard deviation.

Figure 5. The AcMNPV-vp80null mutant is unable to produce any infectious/non-infectious budded virions. The Sf9 cells were independently transfected with 20 µg of bacmid DNA of Ac-Avp80 (a), Ac-wt (b), Ac-Avp80-vp80 (c), Ac-Avp80-pH-vp80 (d), Ac-Avp80-FLAG-vp80 (e), or Ac-Avp80-vp80-FLAG (f). Five days p.t., the budded virus-enriched cell culture supernatants were ultracentrifuged and budded viruses were observed by negative staining electron microscopy (A). The bars represent 200 nm. Parallelly, harvested budded virions were also either separated on SDS-PAGE, blotted and immuno-detected using anti-VP39 antibody or used for PCR-based detection to detect the presence of viral particles (B).

Figure 6. The null bacmid mutant in the vp80 gene forms small number of nucleocapsids, and is deficient in production of occlusion-derived virions. The Sf9 cells transfected either with Ac-Avp80 (A to D), Ac-Δvp80-vp80 (E, F), or
Ac-wt (G, H) were fixed, stained, embedded and thin-sectioned as described in Materials and Methods. (A) Representative overview of Sf9 cell transfected with Ac-vp80null bacmid mutant. (B) The Ac-vp80null mutant does form less number of nucleopsids in the virogenic stroma (C), and no occlusion-derived virions in the ring zone of transfected cells (D). On the other hand, repair bacmid construct Ac-Δvp80-vp80 fully regenerates formation of plenty nuclecapsids in the virogenic stroma (E), as well as normally-apperering occlusion-derived virions in the ring zone of transfected cells (F). Representative images of the virogenic stroma (G) and the ring zone (H) of cells transfected with Ac-wt bacmid. Bars represent 500 nm.

Abbreviations: Nc, nucleocapsid; NM, nuclear membrane; Nu, nucleus; RZ, ring zone; Mi, mitochondrion; ODV, occlusion-derived virions; VS, virogenic stroma.

**Figure 7. Functional complementation of the Ac-vp80null bacmid mutant using the trans-acting vp80 gene.** The Sf9 cells were transfected with either pLZ-flag-vp80 (A) or pLZ (B) vector, and subjected to Zeocin-based selection. Three weeks post-transfection, a polyclonal Zeocin resistant populations of cells were seeded to new 6-well plate and transfected with the Ac-vp80null bacmid mutant to check complementation activity. Virus propagation was monitored by EGFP-specific fluorescence at 72 h and 96 h p.t. At 120 hours p.t., the cell culture supernatants were collected to initiate a secondary infection in untreated (wild-type) Sf9 cells (right panel). EGFP was detected at 72 hours p.i. to signal the progress of infection. EGFP was detected at 120 hours p.i. to signal the progress of infection.

**Figure 8. Construction of an AcMNPV vp39-null bacmid.** (A) Strategy for construction of a vp39-null bacmid containing a partial deletion of the AcMNPV vp39 open-reading frame via homologous recombination in E. coli. At the first step, an internal 498-bp fragment of the vp39 ORF was deleted and replaced with a sequence cassette containing the chloramphenicol (cat) resistance gene flanked by modified loxP (LE and RE) sites. Subsequently, the cat gene was eliminated from the bacmid sequence using the Cre/loxP recombination system. The promoter sequences of the lef-4 and cg-30 genes were not affected. Arrows indicate the positions of oligonucleotide pairs used in PCR analysis of the wild-
type locus and two vp39 knock-out genotypes to confirm the partial deletion of the
vp39 ORF and the correct insertion/deletion of the cat gene cassette. Primers
names are designated according to the nucleotide sequence coordinates. (B) PCR-based detection of the presence or absence of sequence modifications in the
vp39 locus of Ac-wt, Ac-i/p39null(+cat), and Ac-i/p39null(-cat) bacmids. The figure
is showing the PCR-based verification of the correct recombination processes in
the vp39 locus using the 75834/76420 primer pair.

Figure 9. Determination of viral replication capacity of AcMNPV-vp39
 knock out and repaired bacmid constructs using transfection-infection assays (A) Schematic representation of expression cassettes, Tn7-based transposed into the polyhedrin locus. (1) vp39 expressed form the polyhedrin promoter, (2) a double gene vp39 and lef-4, both driven by their native promoters, (3) a double gene vp39 and cg-30 both driven by the polyhedrin promoter, and finally (4) a double gene construct of N-terminally FLAG-tagged vp39 driven by the polyhedrin promoter and the cg-30 ORF expressed from both its native and also the more upstream polyhedrin promoter. The parental bacmid genome backbones used for transfection assays are indicated on the left. The wild type AcMNPV (bMON14272) bacmid was used as positive control of viral replication. (B) Time course fluorescence microscopy showing the propagation of the infection in Sf9 cells transfected with indicated bacmid constructs. Viral progressions were checked by EGFP detection at indicated times post transfection. At 168 hours p.t., the cell culture supernatants were collected to initiate a secondary infection. (C) Secondary infection assay. EGFP detection was performed at 72 hours p.i. to measure progress of the infection.

Figure 10. Construction of an AcMNPV vp1054-null bacmid. (A) Strategy for the construction of a vp1054-null bacmid containing a deletion of the AcMNPV
vp1054 open-reading frame via homologous recombination in E. coli. A 955-bp sequence from the 3'-end of the vp1054 ORF was deleted and replaced with a cat sequence cassette flanked by modified loxP (LE and RE) sites. At the same time, a single point mutation was introduced to change the first translation codon
ATG→Met to ACG→Thr, to prevent translation into a C-truncated VP1 054 protein.
It also meant that the internal AAT codon no. 32 of lef-10 was mutated to AAC, both encoding Asn. Subsequently, the cat gene was eliminated using the Cre/loxP recombination system. The promoter sequence of vp1054/lef-10 was not affected in the bacmid construct. Since the polyadenylation signal of the lef-10 gene was removed, a novel synthetic poly-A signal combined with stop codon (TAATAAA) was introduced at the 3′-end of the lef-10 ORF. Arrows represent locations of oligonucleotide pairs used in the PCR analysis of the wild-type locus and two vp1054 knock-out genotypes to confirm the deletion of the vp1054 ORF and correct insertion/deletion of the cat cassette. (B) PCR-based detection of the presence or absence of sequence modifications in the vp1054 locus of Ac-wt, Ac-i/p7054null(+cat), and Ac-vp7054null(-cat) bacmids. The top figure is showing confirmation of the vp1054 gene deletion and insertion of the cat cassette into vp1054 locus using primer pairs 90292/90889 and cat-F/cat-R. The bottom figure shows CR-based verification of the correct recombination processes in the vp1054 locus using the 89507/91713 primer pair.

Figure 11. Viral replication capacity of AcMNPV-vp7054 knockout and repaired bacmid constructs using transfection-infection assays (A)
Schematic representation of expression cassettes transposed into the polyhedrin locus. The bacmid genome backbones used for transfection assays are indicated on the left. Two Ac-vp7054null-derived constructs were made: first construct carrying only egfp marker gene under control of p10 promoter, and second construct carrying both egfp marker and over-lapping lef-10/vp1054 locus directed from their natural promoter sequences (d). As positive control of viral replication the wild type AcMNPV (bMON14272) bacmid was used (a). The Ac-gp64null bacmid was used as negative control representing a prototype bacmid with a "single-cell infection" phenotype (b). (B) Time course fluorescence microscopy showing the propagation of the infection in Sf9 cells transfected with indicated bacmid constructs. Progress of viral infection was checked by EGFP detection at indicated times post transfection. At 120 hours p.t., the cell culture supernatants were collected to initiate a secondary infection. (C) Secondary infection assay. EGFP was detected at 72 hours p.i. to signal the progress of infection.
Figure 12. Construction of an AcMNPV p6.9-null bacmid. (A) Strategy for construction of a p6.9-null bacmid containing a complete deletion of the AcMNPV p6.9 open-reading frame via homologous recombination in E. coli. A 164-bp fragment of the p6.9 ORF was deleted and replaced with a cat resistance gene flanked by modified loxP (LE and RE) sites. Subsequently, the cat gene was eliminated from the bacmid sequence using Cre/loxP recombination. The promoter sequence of p6.9 gene was left unaffected, since its sequence is overlapping with the p40 ORF. Arrows represent locations of primer pairs used in the PCR analysis of the wild-type locus and two p6.9 knock-out genotypes. (B) PCR-based detection of the presence or absence of sequence modifications in the p6.9 locus of Ac-wt, Ac-vp6.9null(+cat), and Ac-vp6.9null(-cat) bacmids. The top figure shows the insertion of the cat cassette into the p6.9 locus using primer pairs cat-F/cat-R. The bottom figure shows PCR-based verification of the correct recombination processes in the p6.9 locus using the 86596/86995 primer pair.

Figure 13. Viral replication capacity of AcMNPV-p6.9 knockout and repaired bacmid constructs using transfection-infection assays (A) Schematic representation of expression cassettes transposed into the polyhedrin locus. Two repair constructs were made (AcMNPV p6.9 and SeMNPV p6.9 genes, both driven by the AcMNPV p6.9 promoter). The bacmid genome backbones used for transfection assays are indicated on the left. As positive control of viral replication the wild type AcMNPV (bMON14272) bacmid was used. The Ac-gp64null bacmid was used as negative control representing a prototype bacmid with a "single-cell infection" phenotype. (B) Time course fluorescence microscopy showing the propagation of the infection in Sf9 cells transfected with indicated bacmid constructs. Progress of viral infection was checked by EGFP detection at indicated times post transfection. At 120 hours p.t., the cell culture supernatants were collected to initiate a secondary infection. (C) Secondary infection assay. EGFP was detected at 72 hours p.i. to signal the progress of infection. (D) Comparisons of growth curves of AcMNPV-p6.9null (a), AcMNPV-p6.9null rescued with AcMNPV p6.9 (b), and AcMNPV-p6.9null rescued with SeMNPV p6.9 (c) constructs with wild-type (Ac-wt) bacmid. Sf9 cells were transfected with 5.0 µg of DNA from each bacmid, cell culture supernatants were harvested at the indicated
time points post-transfection and analysed for the production of infectious budded virus by an TCID$_{50}$ end-point dilution assay. Infectivity was determined by monitoring EGFP expression. The points indicate the averages of titers derived from three independent transfections, and the error bars represent the standard deviation.

Figure 14: Western blot analysis of Flag:vp80 in cells, BV and ODV
Time course of vp80 expression in infected insect cells. Sf9 cells were infected with the Ac-Δvp80-Flag.vp80 repair virus, and harvested at an indicated time points. Flag-VP8O was detectable by western blot analysis from 12 h to 72 h p.i. as a band of approximately 95 kDa. In addition, a second Flag-VP8O-specific band of -80 kDa accumulated from 48 h till 72 h p.i. Tubulin was used as an internal loading control. (A) The VP80 associates with the nucleocapsid fraction of BV. Two days p.i., BVs were purified by isokinetic ultracentrifugation in a sucrose gradient and separated into nucleocapsid (Nc) and envelope (Env) fractions by Nonidet-P40-based extraction. Flag-VP8O was detected in the Nc fraction as a double-band with molecular weights between the two variants (80-kDa and 95-kDa) detected in infected Sf9 cells (upper panel). Correct separation into Nc and Env fractions was controlled by anti-VP39 and anti-GP64 antibodies (bottom panels). (B) VP80 is also a structural component of ODV- nucleocapsids. Sf9 cells were co-infected with Ac-Δvp80-Flag.vp80 (MOI=25) and AcMNPV strain E2 (MOI=5) viruses. Five days p.i., ODVs were released from occlusion bodies and subsequently separated into nucleocapsid (Nc) and envelope (Env) fractions. Western blot analysis showed that VP80 is present in the DV Nc fraction as a single band of -80 kDa. Proper fractionation into Nc and Env fractions was controlled using anti-PIF-1 antiserum (bottom panel).

Figure 15. Functional complementation of the Ac-vp80null bacmid defective in BV production by trans-complementation. (A) Detection of FLAG:VP80 in a transgenic Sf9-derived cell line (Sf9-vp80) by Western analysis. Tubulin was used as an internal loading control. (B) Time-course fluorescence microscopy (EGFP) to follow the infection in Sf9-vp80 cells transfected (i) or infected (ii) with the Ac-
Δvp80 bacmid (a,b). At 120 h p.t., the cell culture supernatants were collected to initiate a secondary infection in either Sf9-vp80 (a) or Sf9 (b) cells (panels on the right side). As negative control Ac-Δvp80 was propagated in Sf9 cells (c), Ac-wt propagated in Sf9 cells (d) was used as positive control. (C) Comparative release of infectious BV virions. Sf9-vp80 cells were transfected with the Ac-Δvp80 bacmid and Sf9 cells with either the Ac-Δvp80 (negative control) or the Ac-wt (positive control) bacmid. BVs were quantified in cell culture supernatants at 6 days p.t. by end point dilution. Representative results of three independent assays with error bars giving the SD are shown.

**Figure 16. Analysis of foreign gene expression by trans-complemented, replication-deficient baculovirus seed.** Sf9 cells were infected with Ac-wt, Ac-Δvp80-Flag:vp80 or Ac-Δvp80 virus seed (MOI=1 O TCID\textsubscript{50} units per cell), all expressing eGFP from the very late p10 promoter. (A) At 48 h p.i. the presence of EGFP, Flag:VP80 and GP64 was analyzed by Western blotting. Actin was used as an internal loading control. (B) Photomicrographs of cells expressing EGFP 72 h p.i. (top), and relative amount of EGFP measured by ELISA at 48 and 72 h p.i. (bottom) (C) Photomicrographs of cells expressing EGFP 72 h p.i. (top), and analysis of BV release to test for revertant genotypes by TCID\textsubscript{50} titration (bottom). The results of three independent assays are shown with error bars (SD) (B and C).

**Figure 17. The novel baculovirus-insect cell technology approach designated for the production of biopharmaceuticals free of contaminating baculoviral virions.** (A) Insect cell engineering to express an essential viral factor (vp80) to complement a vp80 mutation in the virus. The transgenic Sf9 cells encode the vp80 ORF and a resistance gene allowing antibiotics-based selection of the transgenic cells. (B) Generation of an Ac-Avp80 bacmid defective in production of BV and ODV virions. The bacmid lacks the entire vp80 ORF. (C) Production of a baculovirus seed stock by trans-complementation in the engineered Sf-vp80 cells. The Sf9-vp80 cells are transfected with the Ac-Avp80 bacmid to produce trans-complemented virus progeny. After budded virus propagation, high-titer virus stocks are produced in the Sf9-vp80 packaging cells.
(D) Baculovirus-based recombinant protein expression. Conventional Sf9 cells are infected with the trans-complemented budded virus progeny. Recombinant protein is expressed from very late baculovirus promoters \((p10 \text{ or } polh)\) allowing high levels of expression, while no contaminating baculovirus virions (BV/ODV) are produced.

EXAMPLES

Example I

Materials and Methods

**Insect cells and viruses**

*Spodoptera frugiperda* (Sf9) cells were maintained in SF900-II serum-free medium (Invitrogen) under standard conditions. Recombinant bacmic-dehved AcMNPV virus (ACMNPV-EGFP) carrying an *egfp* reporter gene under control of the very late polyhedrin promoter transposed into the polyhedrin locus was obtained from Pijlman et al. (2006). The virus was propagated and its titers were determined by an end-point dilution assay in Sf9 cells.

**In vitro synthesis of dsRNA**

The method used to synthesize dsRNA is similar to that described by Ramadan et al. (2007) with minor modifications. All DNA templates were PCR amplified using primers with twenty-five nucleotide overhangs homologous to the T7 RNA polymerase promoter sequence \(5'\)-gcttctatacgtacactataggg-3'. The sequences of the primers indicated below are given in Table 1. The following primers were used for amplifying these genes: primers *vp39*-F and *vp39*-R for *vp39*; primers 4551 0 and 46235 for *vp1054*, primers 90292 and 90889 for *vp80*; primers ec-27-F and ec-27-R for *odv-ec27*; and primers dbp-F and dbp- for *dbp*. To test the efficiency of the RNAi studies we made dsRNA against *egfp* with primers *gfp*-F and *gfp*-R, and to have a negative control we made dsRNA with primers *cat*-F and *cat*-R for the chloramphenicol acetyl transferase (*cat*) gene.
The PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and were used as templates for dsRNA in vitro synthesis using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA) according to manufacturer's protocol. Briefly, approximately 1 µg of purified DNA templates were used for RNA synthesis at 37°C for 4 h. After synthesis, DNA templates were removed by digestion with DNase. Complementary RNA strands were annealed by incubation at 70°C for 10 min followed by slow cooling to room temperature (-30 min). Non- annealed (single-stranded) RNA molecules were degraded by RNase A treatment (30 min, 37°C). Finally, the dsRNA was isopropanol precipitated, resuspended in DEPC-treated sterile water to a final concentration of 0.5-1 mg/ml, and its purity and integrity were checked by agarose gel electrophoresis. The dsRNA was kept at -80°C in aliquots of 40 µl. Immediately before transfection, the dsRNA was thawed on ice.

RNAi procedure in baculovirus-infected insect cells

Sf9 cells were seeded in 24-well tissue culture plates (2*10^5 cells/well) in 1 ml Sf900-ll culture medium without serum at 28°C. After two hours, the culture medium was removed, and the cells were infected with recombinant baculovirus ACMNPV-EGFP at a multiplicity of infection (MOI) of 10 TCID_{50} units/cell for 1 h, under standard conditions. One hour post infection (p.i.), dsRNA (20 µg/well) was introduced into the cells by Cellfectin™-based (Invitrogen) transfection in Grace's serum-free medium. After 4 h, the transfection mixture was replaced with Sf900-ll serum-free medium. The cells were incubated for a total of 48 h p.i. at 28°C and then harvested by centrifuging at 1000 x g for 5 min for Western blot and electron microscopy analysis. However, one fifth of the culture medium was harvested at 36 h p.i., and used for titration of budded virions by end-point dilution assays or for PCR-based detection of viral DNA. In all the experiments, dsRNA corresponding to the cat gene was taken as negative control. On the other hand, egfp gene-specific dsRNA was used as positive control for the RNAi procedure.
SDS-polyacrylamide electrophoresis and western blotting

For immuno-detection, the Sf9 cells were disrupted in 125 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, pH 6.8 at 95°C for 10 min. Proteins were separated in 10% SDS-polyacrylamide gels, and subsequently transferred to Immobilon-P membranes (Millipore) by semi-dry electroblotting. Membranes were blocked for 30 min in 1x PBS containing 2% fat-extracted milk powder, followed by incubation for 1 h at room temperature with either rabbit polyclonal anti-GFP antiserum (Molecular Probes), rabbit polyclonal anti-VP39 antiserum, or monoclonal anti-α-tubulin antibody (Sigma-Aldrich), all diluted 1/2000 in 1x PBS containing 0.2% milk power. After washing (3x 10 min) in 1x PBS, the membranes were incubated with 1/4000 dilution of either goat anti-rabbit IgG or rabbit anti-mouse IgG antibodies conjugated with alkaline phosphatase (Sigma). After final washing (3x 10 min) in AP buffer (100 mM Tris-Cl [pH 9.5], 100 mM NaCl, 5 mM MgCl₂), the blots were developed with 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyolphosphate (BCIP) (Bio-Rad) according to the manufacturer’s instructions.

Preparation of viral genomic DNA and its PCR-based detection

Two-hundred microliters of cell culture medium were collected at 36 h p.i. and used for preparation of viral DNA. The cells and cell debris were removed from samples by centifuging at 1000 x g for 5 min. Supernatants containing budded virions were quantitatively transferred to new sterile tubes and centrifuged again at 12000 x g for 90 min. Pelleted BVs were re-suspended in 200 µl TE buffer (10 mM Tris-HCl [pH 7.5], 1mM EDTA) containing Proteinase K (540 µg/ml), and incubated at 55°C for 2 h. A phenol:chloroform:isoamyl alcohol (25:24:1) and a chloroform extraction were subsequently performed. The DNA was precipitated by adding an equal amount of isopropanol and the pellet was washed with 70% ethanol. The DNA pellet was dissolved in 15 µl sterile water, and 2 µl of the final DNA solution was applied to PCR-based detection of the vp39 gene sequence using primers mentioned above. All PCR reactions were performed in 25 µl volumes including: 2 µl DNA, 200 µM dNTPs, 10 pmol of each primer, 1.5 mM MgCl₂ and 1.5 U GoTaq
DNA polymerase (Promega). Amplification conditions were as follows: an initial
denaturation at 94°C for 2 min, after which 30 cycles of denaturation (30 s at
94°C), primer annealing (20 s at 60°C) and primer extension (25 s at 72°C). The
termination cycle was 7 min at 72°C. Negative controls were included in all PCR
amplifications to test for contaminants in the reagents. Aliquots (3.0 µl) of the PCR
products were analysed by electrophoresis in 1.2% (w:v) agarose gels, with 1x
TAE buffer, stained with ethidium bromide (0.5 µg/ml).

Generation of an antibiotic resistance gene-free AcMNPV vp80-KO\bacmid

To determine whether the VP80 protein has an essential role in the context of viral
progeny production, we constructed an AcMNPV bacmid (derived from
bMON14272 (from Invitrogen)) with a deletion of the vp80 ORF by homologous
recombination in E. coli. To accomplish this, a cat gene flanked by mutant LoxP
sites (Suzuki et al., 2005) was amplified using PCR primers vp80-KO-F and vp80-
KO-R (see Table 1) from a plasmid comprising a cat gene flanked by mutant LoxP
sites. The resulting PCR fragment, which contained the cat gene flanked by
mutant LoxP sites and AcMNPV ~50-bp homology sequences to the 5’ or 3’
proximal region of the vp80 ORF, was treated with DpnI and gel-purified to
eliminate the template plasmid. The PCR product was then transformed into
DH1 Oβ E. coli cells containing bMON14272 (Invitrogen) and the Lambda RED
recombinase-producing plasmid pKD46 (Datsenko & Wanner, 2000), which had
been prepared in the following manner. Transformed DH1 Oβ-bMON14272/pKD46
E. coli cells were grown in 50-ml LB (2.0% peptone, 0.5% yeast extract, 85.5 mM
NaCl, [pH 7.0]) cultures with kanamycin (50 µg/ml), ampicillin (100 µg/ml) and L-
arabinose (1.5 mg/ml) at 30°C to an OD₆₀₀ of 0.6 and then made
electrocompetent by a standard procedure. The electroporated cells were
incubated at 37°C for 3 h in 3 ml LB medium and plated on LB-agar containing
chloramphenicol at a concentration of 6.5 µg/ml. After 48-h incubation at 37°C, the
chloramphenicol-resistant colonies were streaked to fresh LB-agar medium with
34 µg/ml chloramphenicol. The plates were incubated at 37°C overnight, and
colonies resistant to chloramphenicol were selected for further confirmation of the
relevant genotype by PCR. Primers 90292 and 90889 were used to confirm the
absence of the vp80 ORF, and primers cat-F and cat-R were employed to verify the presence of cat cassette into bacmid (detailed sequences in Table 1).

To eliminate the introduced antibiotic resistance gene (cat) from the bacmid backbone, a Cre/LoxP recombinase system was employed. A Cre recombinase-carrying plasmid pCRE obtained from Jeanine Louwerse (LUMC Leiden, The Netherlands) was introduced into DH10b-bMON14272-vp80null E. coli cells, and CRE expression was subsequently induced by the addition of isopropyl thiogalactoside (IPTG). Briefly, the electroporated cells were incubated at 37°C for 3 h in 3 ml of LB medium (2.0% peptone, 0.5% yeast extract, 85.5 mM NaCl, [pH 7.0]) and plated on LB-agar medium containing 50 µg/ml kanamycin, 100 µg/ml ampicillin and 2mM IPTG. After 24-h incubation, colonies resistant to kanamycin and ampicillin were selected for further verification of the desired genotype by PCR. In PCR-based analysis, primers 89507 and 91713 (Table 1) were used to verify elimination of cat gene from bacmid backbone. Positive clones were also confirmed by DNA-sequencing.

To recover transposition competence, the helper transposase-encoding plasmid pMON7124 (Invitrogen) was re-introduced into DH10β-bMON14272-vp80null E. coli cells. Finally, the egfp reporter gene was introduced into the vp80-null bacmid to facilitate observation of its behaviour in insect cells. Briefly, the egfp reporter gene was amplified using PCR oligonucleotides gfp-N/?el-F and gfp-Sp/?l-R (Table 1) from plasmid pEGFP-N3 (Clontech). The PCR product was cloned into plasmid pJeti.2/Blunt using CloneJET™ PCR Cloning Kit (Fermentas) according to manufacturer's protocol. Subsequently, the egfp ORF was excised from error-free pJeti.2-egfp with NheI and SphI and subcloned into ΛfTel/SpfI-digested pFastBacDUAL (Invitrogen), to generate plasmid pFB-egfp. An expression cassette containing the egfp reporter gene under transcriptional control of the very late p10 promoter was transposed from pFB-egfp into polyhedhn locus of vp80-null bacmid as described in the Bac-to-Bac manual (Invitrogen). In the resulting genome, the complete vp80 ORF has been removed (see Figure 2). This corresponds to the deletion of 2074 bp from nucleotide positions 89564 to 91637 in the AcMNPV clone C6 genome provided in SEQ ID NO: 1.
Construction of repaired vp80-nu\bacmids

To prepare vp80 repair donor vectors, we modified plasmid pFB-egfp (noted above) by removing the polyhedrin promoter and replacing it with a fragment containing the vp80 promoter region and the vp80 ORF. First, a 2300-bp fragment containing both the vp80 promoter and ORF sequence was amplified using primers pvp80-Sful-F and vp80-Xbal-R (Table 1) from bacmid bMON14272 template, and cloned into vector pJeti .2/Blunt (Fermentas) to form pJeti .2-pvp80-vp80. After DNA sequence verification, the vp80 cassette was excised from pJeti .2-pvp80-vp80 by Stu/I/XbaI double digestion, and then subcloned into Bsf1 107/XbaI-digested and gel-purified pFB-egfp to generate donor plasmid pFB-egfp-pvp80-vp80. Parallely, a donor plasmid pFB-egfp-polh-vp80, where vp80 ORF is driven by the very late polyhedrin promoter (polh) was constructed. To this aim, a 2105-bp fragment carrying the vp80 ORF was amplified using primers vp80-Sacl-F and vp80-Xbal-R (Table 1) and cloned into pJeti .2/Blunt, to generate pJeti .2-vp80. In the final step, the vp80 ORF was cut out (Sac/XbaI) from pJeti .2-vp80, and subloned into Sac/XbaI-digested pFB-egfp, to create pFB-egfp-polH-vp80.

To overcome a problem associated with the inavailabilty of anti-VP80 antibody, FLAG tag decoration (N- and C-terminus fusion) of VP80 was performed to facilitate immunodetection. The N-terminally fused FLAG-vp80 sequence was generated by a double-step PCR strategy, a so-called fusion PCR. First, a 259-bp fragment containing the vp80 promoter and the FLAG tag was PCR amplified using primers pvp80-Sful-F and vp80-FLAG-R1 from the bMON14272 bacmid template. After gel-purification and DNA quantification, the 259-bp fragment was used as forward primer in a second step PCR amplification with the reverse primer vp80-Xbal-R on the bMON14272 bacmid template. The final PCR product (2324 bp) was cloned into vector pJeti .2/Blunt (Fermentas) to form pJeti .2-pvp80-FLAG-vp80. After DNA sequence verification, the FLAG-vp80 cassette was excised from pJeti .2-pvp80-FLAG-vp80 by Stu/I/XbaI double digestion, and then subcloned into Bsf1 107/XbaI-digested and gel-purified pFB-egfp to generate donor plasmid pFB-egfp-pvp80-FLAG-vp80. The C-terminally fused vp80-FLAG cassette was amplified using pvp80-Sful-F and vp80-FLAG-R from the bMON14272 bacmid.
template. The 2324-bp fragment was cloned into pJet 2.2/Blunt, and subsequently transfer into pFB-egfp in a similar way as previous constructs.

The inserts of all developed donor plasmids were transposed into the vp80-null bacmid following the Bac-to-Bac protocol (Invitrogen). Screening of transposition-positive constructs into the polh locus was done by a the triplex PCR-based assay employing a M13 forward and reverse primers and a gentamicin resistance gene-specific primer GenR (Table 1).

**Transfection-infection assay**

Bacmid DNAs were prepared from 1.5-ml over-night bacterial cultures of 2 to 3 independent colonies carrying the bacmid with the inserted heterologous gene according to the Bac-to-Bac manual (Invitrogen) and were analyzed in parallel. For transfections, 1 µg of each bacmid DNA preparation was used to transfect 1x10^6 Sf9 cells in a 6-well plate by the Cellfectin™-based transfection protocol as described in the Bac-to-Bac (Invitrogen) manual. From 72 h to 120 h post transfection (p.t.), viral propagation was checked by fluorescence microscopy. At 120 h p.t., the cell culture medium was centrifuged for 5 min at 2000 x g to remove cell debris, and this clarified supernatant was used to infect 1.5x10^6 Sf9 cells in 6-well plates. After 72 h p.i., the spread of virus infection was again monitored by fluorescence microscopy. In all experiments, a wild-type bMON14272 bacmid carrying the egfp reporter gene under control of the p10 promoter was used as positive control. A bMON14272-gp64null bacmid also carrying the egfp reporter gene under control p10 promoter served as negative control, since it has lost the ability of cell-to-cell movement of the infection (Lung et al., 2002).

**Time-course characterization of viral propagation in cell culture**

Time course analyses were performed to compare budded virus production of the AcMNPV-vp δOnull virus and the various repair constructs in comparison to the wild type AcMNPV bacmid (Ac-wt) all containing egfp. Briefly, the Sf9 cells were seeded in 6-well tissue culture plates (1x10^6 cells/well in 1 ml Sf900-II culture medium without serum at 28°C. After two hours, the culture medium was removed, and the cells were transfected with 5 µg bacmid DNA, under standard conditions as recommended in Bac-to-Bac manual (Invitrogen). Cell culture supernatants
were harvested at 24, 48, 72, 96 and 120 h p.t., and analysed for the production of infectious budded virus by an end-point dilution assay to determine the tissue culture infective dose 50 (TCID$_{50}$). Infection was determined by monitoring egfp expression (from the p10 promoter). The average values of infectious titers derived from three independent transfections were calculated and plotted into graphs.

Transmission electron microscopy
Insect Sf9 cells were seeded in 25T flask (3.5x10$^6$ cells/flask), and transfected with 20 µg either the Ac-Δvp80, rescue Ac-Δvp80-vp80 or Ac-wt bacmid construct. After 48 h p.t., the cells were harvested and prepared for transmission electron microscopy as described previously (van Lent et al., 1990). Samples were examined and photographed with a Philips CM12 electron microscope.

Budded virus production assay
Insect Sf9 cells were seeded in two 25T flasks (3.5x10$^6$ cells/flask), and transfected with 20 µg either Ac-Avp80, Ac-Avp80-vp80, Ac-Avp80-pH-vp80, Ac-Avp80-FLAG-vp80, Ac-Avp80-vp80-FLAG, or Ac-wt bacmid construct. Five days p.t., the BV-enhched cell culture supernatants were harvested, and ultracentrifuged through a cushion of 10% sucrose solution (25,000 rpm for 1.5 hour, Beckman SW32). Pelleted budded virions were resuspended in sterile demi-water, and prepared for either negative staining electron microscopy, SDS-polyacrylamide electrophoresis, or PCR-based detection (as mentioned the above).

Purification of ODVs and rod-shaped structures from infected cells
The presence of ODVs and rod-like structures in infected/transfected insect cells was analyzed by electron microscopy (EM). For this purpose, insect cells were harvested 48 h p.i., lysed and the cell lysates were ultracentrifuged through a 40% sucrose cushion in TE (1 mM Tris-HCl pH 7.4, 0.1 mM EDTA) buffer (45,000 rpm for 1 hour, Beckman SW55). Pellets were resuspended in sterile demi-water and analyzed by negative staining EM as described previously (van Lent et al., 1990).

Development of transgenic Sf9-derived cell line expressing vp80
To develop a cell line, which produces the VP80 protein, a 2105-bp fragment carrying the vp80 ORF was amplified using primers vp80-Sacl-F and vpδO-Xbal-R (Table 1) and cloned into pJeti .2/Blunt, to generate pJeti .2-vp80. In the next step, the vp80 ORF was cut out (SacI/XbaI) from pJeti .2-vp80, and subcloned into Sacl/XbaI-digested plZ (Invitrogen), to create plZ-vp80. The resulting plasmid vector plZ-vp80 was linearized with Eco57I, and gel-purified. Sf9 cells were seeded in six-well plate (1x10^6 cells/well), and transfected with 10 µg of the linearized vector. After 24 hours post-transfection, cells were selected by cell culture medium containing Zeocin™ (300 µg/ml) for 2 to 3 weeks, until no control Sf9 cells survived under the same conditions. Cells were then propagated as an uncloned cell line.

**Generation and characterization of a AcMNPV vp39-nu\bacmid**

To study the role of vp39 gene in the context of viral progeny production and the nucleocapsid assembly process, we constructed an AcMNPV bacmid (bMON14272) with a deletion of vp39 by homologous recombination in E. coli according to the same procedure as noted above for the AcMNPV vp80r\bacmid bacmid construct. Since the sequence of the vp39 ORF is overlapping with promoter sequences of both flanking ORFs (cg-30 and lef-4), only an internal part of the vp39 ORF could be deleted, to avoid de-regulations of cg-30 and lef-4 expression. To reach this, a cat gene flanked by mutant LoxP sites was amplified using PCR primers vp39-KO- and vp39-KO-R (Table 1) from a plasmid comprising a cat gene flanked by mutant LoxP sites. The resulting PCR fragment, which contained the cat gene flanked by mutant LoxP sites and ~50-bp sequences homologous to an internal region of the vp39 ORF, was treated with Dpn\ and gel-purified to eliminate the template plasmid. The PCR product was then transformed into DH1 0β E. coli cells containing bacmid bMON14272 (Invitrogen) and Lambda RED recombinase-producing plasmid pKD46 (Datsenko & Wanner, 2000) prepared in the above mentioned manner. In the final step, colonies resistant to kanamycin were subjected to PCR-based analysis using primers 75834 and 76420 (Table 1) to verify insertion/elimination of the cat gene from the bacmid backbone. Positive clones were further verified by DNA-sequencing of the obtained PCR
products. According to this protocol, an internal part (498 nt = 166 aa) of the vp39 ORF was removed, coordinates: 75894-76391 as indicated in Figure 9.

Construction and analysis of repaired \textit{vp39-nu} bacmids

To prepare a \textit{vp39} repair donor vector, we modified plasmid pFB-egfp (noted above) by introduction of the \textit{vp39} ORF under control of the polyhedrin promoter. Initially, a 1073-bp fragment was amplified using primers \textit{vp39-Sacl-F} and \textit{vp39-Xbal-R} (see Table I for primer sequences) from the bMON14272 template, and cloned into vector pJeti \textit{.2/Blunt} (Fermentas) to form pJeti \textit{.2-vp39}. After DNA sequence verification, the \textit{vp39} ORF was excised from pJeti \textit{.2-vp39} by \textit{SacI/XbaI} double digestion, and then subcloned into \textit{Sacl/XbaI}-digested and gel-purified pFB-egfp to generate donor plasmid pFB-egfp-vp39. After an unsuccessful attempt to rescue AcMNPV \textit{vp39-nu} with pFB-egfp-vp39, a set of novel donor plasmids was prepared. First, a 2498-bp fragment containing \textit{vp39} and \textit{lef-4} ORFs was PCR-generated using primers \textit{vp39-Sful-F} and \textit{lef-4-Xbal-R} from bacmid bMON14272 template, and cloned into vector pJeti \textit{.2/Blunt} (Fermentas) to form pJeti \textit{.2-vp39-lef-4}. After DNA sequence confirmation, the fragment containing \textit{vp39} and \textit{lef-4} ORFs was excised from pJeti \textit{.2-vp39-lef-4} by \textit{Sful/XbaI} double digestion, and then subcloned into Sful/XbaI-digested and gel-purified pFB-egfp to generate donor plasmid pFB-egfp-vp39-lef-4.

Parallely, donor plasmid pFB-egfp-vp39-cg30 was constructed, where both \textit{vp39} and \textit{cg-30} ORFs are driven from the very late polyhedrin promoter, and the \textit{cg-30} ORF can also use its native promoter situated inside the 3’-end of the \textit{vp39} ORF. Briefly, a 1868-bp fragment carrying both \textit{vp39} and \textit{cg-30} ORFs was amplified using primers \textit{cg30-Xbal-F} and \textit{vp39-Xbal-R} (noted above) and cloned into pJeti \textit{.2/Blunt}, to generate pJeti \textit{.2-vp39-cg30}. The \textit{vp39/cg-30} cassette was subcloned as \textit{SacMXba} into pFB-egfp, to create pFB-egfp-vp39-cg30. Additionally, a similar donor vector pFB-egfp-FLAG-vp39-cg30 was constructed, where \textit{vp39} ORF is N-terminally FLAG-tagged. The same strategy was employed to develop this vector, only the reverse primer \textit{vp39-FLAG-Sacl-R} was used to amplified \textit{vp39/cg-30} cassette instead of the \textit{vp39-Xbal-R} primer.

All developed donor plasmids were transposed into \textit{vp39-nu} bacmid following the Bac-to-Bac kit protocol (Invitrogen) and screened as detailed above.
for vp80 repair bacmids. The functional analysis was performed as described above for the vp80 constructs.

**Generation and analysis of AcMNPV vp1054-null bacmid**

To verify the essential role of the vp1054 gene in the context of viral progeny production and nucleocapsid assembly, we constructed an AcMNPV bacmid (bMON14272) with a deletion of vp1054 by homologous recombination in E. coli according to the same procedure as for the vp80rnull bacmid construct with minor alternations. Since the vp1054 ORF is overlapping with the essential lef-10 ORF, we could not remove the whole vp1054 ORF, but only a 955-bp nucleotide 3'-end part of the ORF. To prevent translation of the C-truncated VP1054 mutant in insect cells, we decide to mutate the first translation codon ATG→Met to ACG→Thr. This single nucleotide substitution did also change an internal codon no. 32 (AAT) to AAC of lef-10 ORF, however, both are encoding the same amino acid (Asn). To accomplish this, we first amplified the 5'-end of the vp1054 ORF using primers vp1054-KO-F and vp1054-KO-R1 from bacmid bMON14272 (Invitrogen). The 214-bp PCR product contained a mutation of the ATG start codon of the vp1054 ORF, introduced a synthetic stop/poly-A signal sequence for the lef-10 ORF, and has a 3'-end sequence homology overhang to the cat cassette to facilitate the second PCR, and a 49-bp homology sequence to the 5'-end of vp1054 ORF to mediate Lambda RED-directed homologous recombination in E. coli. After gel-purification and DNA quantification, the 214-bp fragment was used as forward primer in a second step PCR with reverse primer vp1054-KO-R2 with a plasmid comprising a cat gene flanked with mutant LoxP sites as template. The resulting 1230-bp PCR fragment, which contained the cat gene flanked by mutant LoxP sites, a mutated 5'-end of the vp1054 ORF and ~50-bp sequences homologous to the 5'or 3' proximal region of the vp1054 ORF, was treated with DpnI and gel-purified to eliminate the template plasmid. Recombination of this PCR product with the bMON14272 bacmid was performed as described above for the vp80 mutant. Kanamycin resistant colonies were verified by PCR with primer pairs cat-F/cat-R, 4551 0/46235, and 451 22 and 46441 to check the insertion/elimination of the cat gene from the bacmid backbone. Insertion sites were also confirmed by DNA-sequencing. This method resulted in the deletion of 955 bp from nucleotide
Construction of a repaired \textit{vp1054-\textbackslash nu} bacmid construct

To prepare \textit{vp1054} repair donor vector, we modified plasmid pFB-egfp (noted above) by removing the polyhedrin promoter and replacing it with a fragment containing the \textit{vp1054} promoter region and the \textit{vp1054} ORF. First, a 1714-bp fragment containing both the \textit{vp1054} promoter and ORF sequence was amplified using primers \textit{vp1054-Rep-F} and \textit{vp1054-Rep-R} from bacmid bMON14272 template, and cloned into vector pJet1.2-Blunt (Fermentas) to form pJet1.2-pvp1054-vp1054. After DNA sequence verification, the \textit{vp1054} cassette was excised from pJet1.2-pvp1054-vp1054 by \textit{Stu\slash XbaI} double digestion, and then subcloned into Bsf11071/XbaI-digested and gel-purified pFB-egfp to generate donor plasmid pFB-egfp-pvp1054-vp1054. The developed donor plasmids were transposed into the \textit{vp1054-\textbackslash nu} bacmid following the Bac-to-Bac protocol (Invitrogen) and screened. Recombinant bacmids were analyzed as detailed above for \textit{vp80} bacmids.

Generation and analysis of AcMNPV \textit{p6.9-null} bacmid

To verify the essential role of \textit{p6.9} in the context of viral progeny production, we constructed an AcMNPV bacmid (bMON14272) with a deletion of \textit{p6.9} by homologous recombination in \textit{E. coli}. To accomplish this, a chloramphenicol resistance gene (\textit{cat}) flanked by mutant \textit{LoxP} sites was amplified using PCR primers p6.9-KO-F and p6.9-KO-R from a plasmid comprising a this \textit{cat} gene flanked by mutant \textit{LoxP} sites. Mutant viruses were obtained following the same procedure as for the other mutants. For the PCR-based analysis of the finally obtained mutant clones the primer pairs cat-F and cat-R and 86596 and 86995 were used to check insertion/elimination of \textit{cat} gene from bacmid backbone. Positive clones were also confirmed by DNA-sequencing. This method results in the deletion of 164 bp from nucleotide positions 86716 to 86879 in the AcMNPV clone C6 genome provided in SEQ ID NO: 1. Table 1 for primer sequences.

Construction and functional analysis of repaired \textit{p6.9-null} bacmids
To prepare p6.9 repair donor vectors, the pFB-GFP-p6.9 vector was used, which was constructed by Marcel Westenberg (Wageningen University). To make this vector, the AcMNPV p6.9 promoter sequence was amplified from the plasmid pAcMPI (Hill-Perkins & Possee, 1990) with primers pp6.9-F and pp6.9-R using the high-fidelity Expand long-template PCR system (Roche). The PCR product was cloned as SalI fragment into pFastBad (Invitrogen), from which the polyhedrin promoter was deleted in advance by fusing the SstI 107I to the StuI site, to obtain pFB1-p6.9. The p6.9 promoter from pFB1-p6.9 was recloned as SnaBl/SamHI fragment into the SstI 107I and BamHI sites of pFastBacDUAL (Invitrogen), thereby deleting the polyhedrin promoter. Subsequently, the egfp reporter gene was cloned downstream of the p10 promoter into the XmaI site to obtain pFB-GFP-p6.9. Finally, the p6.9 genes of AcMNPV and Spodoptera exigua (Se)MNPV were PCR amplified from either the AcMNPV bacmid (bMON14272) or SeMNPV genomic DNA by using the high-fidelity Expand long-template PCR system and primers generating EcoRI and NotI at the 5’ and 3’ ends, respectively (Table 1). The PCR products were cloned downstream of the p6.9 promoter in the EcoRI/NotI sites of pFB-GFP-p6.9. All generated clones were sequenced to verify the incorporated p6.9 sequences.

The expression cassettes of both developed donor plasmids were transposed into the p6.9-null bacmid following the Bac-to-Bac protocol (Invitrogen). Screening of transposition-positive constructs into the polh locus was done by the triplex PCR-based assay as described above for the vp80 constructs. The analysis was performed as for the vp80 constructs.

Results

Silencing of AcMNPV vp80 does not affect baculovirus very late gene expression

We explored the effect of transfecting Sf9 cells with different dsRNAs during infection with AcMNPV-GFP. To trigger dsRNA-induced silencing of selected baculoviral genes (vp1054, vp39, vp80, dbp and odv-ec27), we generated gene-specific dsRNAs using in vitro T7 RNA polymerase-based synthesis. However, when we began these studies it was not clear what amount and time point of
dsRNA transfection is the most effective to silence baculoviral genes. To determine an optimal amount of dsRNA for RNAi assay purpose in baculovirus-infected cells, we first attempted to silence reporter egfp gene with different amounts of dsRNA. These pilot assays showed that the most potent RNAi effect is achieved using 100 pg dsRNA per cell (data not shown). At the same time, it was also proved that RNAi treatment has no negative effect on the production of infectious budded virions progeny. We also tried to transfect dsRNA into the cells at two different time points, 24 h prior to infection or 1 h p.i. The results proved that transfection performed at 1 h p.i. is more efficient in silencing of genes expressed at late/very late phases of baculoviral infection in contrast to transfection carried out at 24 h prior to infection (data not shown). In addition, to ensure that knock-down was gene-specific, dsRNA corresponding to the cat gene was transfected as an RNAi negative control. Herein, we could observe a moderate inhibition of baculovirus infection propagation in comparison to untransfected insect cells. However, the same phenomenon was also observed when insect cells were treated only with transfection reagents. Therefore, we could conclude that the effect can be explained by a negative impact (cytotoxicity) of the presence of transfection reagents on cell viability.

Silencing screening of baculovirus genes revealed that down-regulation of vp1054, vp39, dbp and odv/ec-27 is also associated with a reduction or inhibition of very late gene expression measured by EGFP detection (Fig. 1A and 1B). The highest levels of this inhibition were observed in dbp- and odv/ec-27-targeted cells. The cause of this effect can be explained by the presence of bi-cistronic and overlapping mRNA transcripts, which are produced during a baculovirus replication cycle. Eventually, a cross-reaction with targets of limited sequence similarities can also be involved in the process. Only cells treated with vp80 dsRNA showed a similar level of EGFP expression as untransfected cells or particularly with cat dsRNA-treated cells. Importantly, very few EGFP-producing cells were observed in insect cells where egfp-specific dsRNA was introduced (positive RNAi control), showing that the transfection efficiency was high. Based on our RNAi screening achievements, the vp80 gene (locus) seems to be a suitable candidate for RNAi-based targeting in context of interference with baculoviral very late gene expression.
Knock-down of \textit{vp80} totally prevents production of BVs and normally appearing ODVs

To determine the roles of selected candidate genes \textit{(vp1054, vp39, vp80, dbp and odv/ec-27)} in production of budded virions progeny, cell culture medium (36 h p.i.) from dsRNA-treated cells was examined for the presence of BVs. End-point dilution-based titrations confirmed that all tested genes are essential for infectious budded virus progeny production (Fig. 1C). We were not able to detect any infectious BVs in \textit{vp80}- and ctep-targeted cells. In addition, PCR-based assay indicated that defective or non-infectious viral particles are also not produced in \textit{vp80}-targeted cells. It is important to point out that the results also showed a significant decrease in the production of infectious BVs in the RNAi controls \textit{(egfp- and caf-specific dsRNA-treated cells)} compared to untransfected cells. The cytotoxicity of transfection reagents is again the assumed cause of this negative effect. Electron microscopy analysis of cell lysates showed that formation of ODVs and rod-like structures was totally inhibited in cells treated with dsRNA-vp39 as expected (Fig. 1D). Production of ODVs and rod-like structures was also significantly reduced in insect cells treated with dsRNA-vp80 (Fig. 1D). However, in \textit{vp80}-targeted cells we could mostly find nucleocapsids of aberrant phenotypes (pointed shape). On the other hand, introduction of dsRNA-caf into insect cells did not cause any changes in the production of ODVs.

The AcMNPV \textit{vp80} gene is essential for viral replication

An AcMNPV deletion virus was constructed as detailed in Fig. 2. Repair constructs were designed such that the wild-type \textit{vp80} ORF or N- and C-terminally FLAG-tagged \textit{vp80} genes along with its native or polyhedrin promoter regions were inserted into the polyhedrin locus along with the \textit{egfp} gene under the \textit{p10} promoter (Fig. 3A). To investigate the function of the \textit{vp80} gene, Sf9 cells were transfected with either the knock-out or repair bacmid constructs and monitored for EGFP expression by fluorescence microscopy. When \textit{Ac-vp80} null was introduced into Sf9 cells, no viral propagation was observed in cell culture at 72 h to 120 h p.t. We could observe only a "single-cell infection" phenotype similar to the phenotype of Ac-gp64null bacmid (Fig. 3B). The results indicate that Ac-vp80null is able to
reach the very late phase of infection as confirmed by p10 promoter-driven EGFP expression. From 72 h to 120 hours p.t., widespread EGFP expression could be seen in insect cell monolayers that were transfected with the three repair (vp80 driven from its native promoter, vp80 driven from polyhedrin promoter and N-terminally FLAG-tagged vp80 driven from its native promoter) constructs indicating that these bacmids were able to produce levels of infectious budded virions sufficient to initiate secondary infection at similar level as the wild-type bacmid (Fig. 3B). In contrast, in insect cells transfected with C-terminally FLAG-tagged vp80 repair constructs, by 72 h p.t. EGFP expression was only observed in isolated cells that were initially transfected indicating that this bacmid construct is defective in viral replication (Fig. 3B). However, by 96 h p.t. formation of tiny plaques was observed and by 120 h p.t. very few plaques of normal size were developed. The results show that the C-terminal flagged mutant is strongly delayed in producing budded virus and showed that an unmodified C-terminus is very important for the function of VP80. At 5 days p.t., cell culture supernatants were removed and added to freshly plated Sf9 cells and then incubated for 3 days to detect infection by virus generated from cells transfected with these bacmids. As expected, Sf9 cells incubated with supernatants from the transfections with repair constructs showed numerous EGFP expressing cells (Fig. 3C). Nevertheless, cells incubated with supernatant from C-terminally FLAG-tagged constructs showed a significant reduction in the number of EGFP-positive cells. On the other hand, in insect cells incubated with supernatant from the transfection with the vp80 knockout, no EGFP expression was detected at any time-point analyzed up to 72 h (Fig. 3C).

Moreover, to characterize the exact effect of deletion of the vp80 gene on AcMNPV infection, the viral propagation in transfected Sf9 cells was compared between Ac-wt, Ac-Δvp80, Ac-Δvp80-vp80Rep, Ac-Δvp80-polh-vp80Rep, Ac-Δvp80-FLAG-vp80Rep and Ac-Δvp80-vp80-FLAGRep. Cell culture supernatants of all the above bacmid constructs were analysed at indicated time points for BV production (Fig. 4). As expected, the repaired Ac-Δvp80-vp80Rep, Ac-Δvp80-polh-vp80Rep, Ac-Δvp80-FLAG-vp80Rep viruses showed kinetics of viral replication consistent with wild-type virus (Ac-wt) propagation. Budded virion production by
the C-terminal flagged Ac-Δvp80-vp80-FLAGRep virus was reduced to approximately 0.06% compared to the Ac-wt virus or the other repaired viruses.

These results indicate that the *vp80* gene is essential for infectious BV production. It has clearly been proven that the whole sequence of *vp80* ORF can completely be deleted from the bacmid backbone and adequately rescued by introduction of the *vp80* ORF into a heterologous site (polyhedrin locus) of the genome. We also showed that *vp80* gene expression can be driven by the heterologous polyhedrin promoter sequence with no negative effect on viral replication in cell culture. Additionally, we observed that the N-terminus in contrast to the C-terminus of VP80 is permissive to gene modifications (epitope tag-labeling). We noted that the kinetics of the C-terminally FLAG-tagged VP80 virus was significantly delayed when compared with all other rescue or wild-type viruses, indicating the functional importance of the VP80 C-terminus.

**VP80 is required for production of both BV and ODV**

The results described above indicated that the Ac-vp80null mutant is completely defective in production of infectious budded virus. However, there was also a possibility that the mutant can still produce non-infectious budded particles. To investigate the ability, Sf9 cells were transfected with either the knock-out, repair or wild-type bacmid constructs and 7 days p.t. cell culture mediums were ultracentrifuged to pellet budded viruses. The formed pellets were either analyzed by negative staining electron microscopy or by Western blot- and PCR-based detection to confirm the presence of the budded viruses. No intact budded virus, virus-like particles, nor its structures (such as major capsid protein VP39 and viral genome sequence) were revealed in the pellet from the cells transfected with the Ac-vp80null mutant (Fig. 5A and 5B). On the other hand, all analyzed repair constructs produced normally-appearing budded virus as compared with budded virus-derived from the wild-type virus (Fig. 5A). Nevertheless, it was very difficult to find representative budded virions in the pellet derived from C-terminally FLAG-tagged *vp80* gene repair construct-transfected cells.
To further characterize deletion of the \textit{vp80} gene on baculovirus life cycle, electron microscopy was performed with ultra-thin sections generated from bacmid-transfected cells. The Ac-\textit{vp80null}-transfected cells developed typically phenotype of baculovirus-infected cell with an enlarged nucleus, a fragmented host chromatin, an electron-dense virogenic stroma, etc. (Fig. 6A). The absence of VP80 did not prevent formation of normally-appearing nucleocapsids inside the virogenic stroma (Fig. 6C). The formed nucleocapsids were phenotypically undistinguishable from those produced by either the Ac-\textit{i/p80null} repair or Ac-\textit{wt} bacmids. However, an abundance of assembled nucleocapsids was rather less as compared with cells-transfected with the Ac-\textit{vp80null} repair or Ac-\textit{wt} bacmids (Fig. 6E and 6G). In addition, no occlusion-derived virions nor bundles of nucleocapsids prior to an envelopment could be observed in the peristomal compartment of a nucleoplasm (so called the ring zone) of Ac-\textit{vp80null} bacmid-transfected cells (Fig. 6B and 6D). It seems that VP80 plays a role during maturation of nucleocapsids and/or their release/transport from the virogenic stroma. Eventually, VP80 can somehow contribute to an efficient nucleocapsid assembly which could be explained by small number of nucleocapsids present in the virogenic stroma of Ac-\textit{vp80null} transfected cells. When the \textit{vp80} gene was re-introduced back into the bacmid mutant, a lot of nucleocapsids and occlusion-derived virions could be seen in the ring zones of transfected cells (Fig. 6F). An abundance and morphology of occlusion-derived virions produced in Ac-\textit{Δvp80-vp80} repair bacmid-transfected cells were similar to those produced by wild-type bacmid (Fig. 6F and 6H).

\textbf{VP80 function can be complemented by the trans-acting \textit{vp80} gene}

To prove that VP80 function can be complemented by the trans-acting \textit{vp80} ORF, a complementation assay was performed with a transgenic cell line, Sf9-vp80, that was stably transformed with the \textit{vp80} gene expressed under control of an early baculovirus \textit{Orgyia pseudotsugata} ie-2 promoter. In the assay, both Sf9 and Sf9-vp80 cells were transfected with the Ac-\textit{vp80null} bacmid mutant (Fig. 7). Virus infection spread was monitored by EGFP-specific fluorescence at 72 h and 96 h p.t. In Sf9-vp80 cells we could observe viral plaques demonstrating the virus spread. On the other hand, in Sf9 cells only "single-cell infection" phenotype could be seen as previously described above. After six days, the cell culture
supernatants were harvested and used as a inoculum to infect fresh groups of Sf9 cells. After 5 days, EGFP-positive cells were monitored by fluorescence microscopy. Only "single-cell infection" phenotype was observed in Sf9 cells receiving the supernatant from Sf9-vp80 cells. As assumed, no EGFP signal was detected in Sf9 cells receiving the supernatant from Sf9 cells. These results show that the Ac-vp80null can be rescued by VP80-expressing cells (Sf9-vp80) and demonstrate that the observed complementation is due to VP80 protein expressed from the host cell line and not from acquisition of the vp80 gene from the cell line. In other words, the results match requirements asked to produce biopharmaceuticals (EGFP protein in our model assay) without contaminating baculovirus virions.

**Generation and characterization of vp39-\(\text{nu}\)\| bacmid**

To study the functionality of the AcMNPV vp39 gene during virus infection, an \(\text{vp39-riu}\|\) AcMNPV bacmid was constructed by partial deletion of the \(\text{vp39}\) gene. The deletion construct was selected by its resistance to chloramphenicol indicating that site-specific deletion of the \(\text{vp39}\) gene had occurred. In the resulting \(\text{vp39-riu}\|\) AcMNPV bacmid, the internal part of \(\text{vp39}\) gene was correctly replaced by the \(\text{cat}\) gene. Subsequently, the \(\text{cat}\) was eliminated by \(\text{Cre}/\text{LoxP}\) recombination (Fig. 8A). The \(\text{vp39}\) sequence was removed from nucleotides 75894 to 76391 according to the AcMNPV clone C6 genome sequence (SEQ ID NO:1). The structure of the \(\text{vp39}\) deletion constructs was confirmed by PCR using primers 75834 and 76420 (Fig. 8B). A 647-bp DNA fragment was amplified when wild-type AcMNPV bacmid was used as a template, whereas a 1113-bp DNA fragment could be amplified on AcMNPV \(\text{vp39-nu}\|(+\text{cat})\) template (Fig. 8B). When the final construct AcMNPV-\(\text{vp80nu}\|(-\text{cat})\) with eliminated \(\text{cat}\) cassette was used in PCR analysis, only a short 183-bp DNA fragment could be detected (Fig. 8B). The results were confirmed by DNA sequencing.

**Functional mapping of \(\text{vp39}\) ORF indicates a presumable functional relationship between \(\text{vp39}\) and \(\text{cg-30}\) ORFs**
The repair constructs were designed in such a way that the wild-type vp39 ORF under control of the polyhedrin promoter sequence was inserted into the polyhedrin locus along with the egfp gene controlled by the p10 promoter (Fig. 9A). To investigate the function of the vp39 gene, Sf9 cells were transfected with either the knock-out or repair bacmid constructs and monitored for EGFP expression by fluorescence microscopy. When Ac-vp39 null was introduced into Sf9 cells, no viral propagation was observed from 72 h to 168 h p.t. We could observe only a "single-cell infection" phenotype similar to the phenotype of the Ac-gp64null bacmid (Fig. 9B).

These results indicate that the Ac-i/p39null construct is able to reach the very late phase of infection as shown by the p10 promoter-driven EGFP expression. Unexpectedly, no viral propagation could be seen in insect cell monolayers that were transfected with the vp39 repair (vp39 driven from polyhedrin, Ac-Δvp39-polh-vp39Rep) constructs (Fig. 3B). For this reason, we decided to prepare three extra repair bacmids carrying both vp39 and lef-4 ORFs under controls of their native promoters. When the insect cells were transfected with these repair constructs again viral replication did not occur (Fig. 9B) and a "single-cell infection" phenotype was observed from 72 h to 168 h p.t. Interestingly, in insect cell monolayers that were transfected with the repair constructs carrying both vp39 (or FLAG-tagged vp39) and cg-30 we could observe tiny clusters of EGFP-positive cells (3-5 cells) (Fig. 9B). However, we did not see a full-value viral replication as that of the wild-type vector (Ac-wt).

At 7 days p.t., cell culture supernatants were collected and added to freshly plated Sf9 cells, which were then incubated for 3 days to detect infection by virus generated from cells transfected with all bacmids mentioned here (Fig. 9C). As expected, Sf9 cells incubated with the supernatant from Ac-wt transfections, showed numerous EGFP expressing cells. On the other hand, cells incubated with supernatants from Ac-Δvp39-polh-vp39Rep and Ac-Δvp39-vp39-lef-4Rep constructs did not show any EGFP-positive cells. However, in insect cells incubated with supernatants from Ac-Δvp39-vp39-cg30Rep and Ac-Δvp39-FLAG-vp39-Rep, a number of EGFP-expressing cells was detected (Fig. 9C). These
results indicated that a possible functional relationship between the \textit{vp39} and \textit{cg-30} ORFs is required for baculovirus replication.

Since the \textit{vp39} ORF sequence overlaps with the promoter sequences of the two flanking ORFs (\textit{lef-4} and \textit{cg-30}), we could not delete the whole \textit{vp39} ORF in our \textit{vp39
\textit{nu}}\textit{i} bacmid construct. It may therefore also be that C- and/or N-truncated mutant(s) of \textit{vp39} may be expressed which may interfere as a competitive inhibitor with the normal VP39 protein.

\textbf{Construction and analysis of \textit{vp1054-nu}}\textit{i} bacmid} 

To study the functionality of the AcMNPV \textit{vp1054} gene during virus infection, an \textit{vp1054-nu}}\textit{i} AcMNPV bacmid was constructed by partially deleting the \textit{vp1054} gene from AcMNPV bacmid (bMON14272) by homologous recombination in \textit{E. coli}. The deletion construct was selected by its resistance to chloramphenicol that indicated that site-specific deletion of the \textit{vp1054} gene had occurred. In the resulting \textit{vp1054-nu}}\textit{i} AcMNPV bacmid, the 955-bp 3'-end part of the \textit{vp1054} gene was correctly replaced by the \textit{cat} gene. Subsequently, the antibiotic resistance cassette (\textit{cat}) was eliminated from bacmid backbone using Cre/LoxP recombination system (Fig. 10A). The deleted sequence was removed from the nucleotide coordinates 45365 to 46319 according to the AcMNPV clone C6 genome sequence (SEQ ID NO:1). The structure of all the deletion constructs was confirmed by PCR (Fig. 10B). When the \textit{vp1054} gene is present, as in the parental wild-type AcMNPV bacmid, a 775-bp PCR product can be amplified using primers 45510 and 46235, whereas a 596-bp PCR fragment amplified with cat-F and cat-R primers is produced only when \textit{cat} gene was introduced into bacmid sequence in case of AcMNPV \textit{i/p7054null(+cat)} construct (Fig 10B). Correct recombination process was also confirmed by PCR mapping of \textit{vp1054} locus using primers 45122 and 46441. A 1320-bp DNA fragment was amplified when wild-type AcMNPV bacmid was used as a template, whereas a 1353-bp DNA fragment could be amplified on AcMNPV \textit{vp1054-nu}}\textit{i(+cat)} template (Fig. 10B). When final construct AcMNPV-\textit{i/p7054null(-cat)} with eliminated \textit{cat} cassette was used in PCR analysis, only a 423-bp DNA fragment could be detected (Fig. 10B). Positive clones were successfully verified by DNA sequencing.
**AcMNPV vp1054 gene is essential for viral replication**

The repair construct was designed such that the AcMNPV vp1054 ORF with its native promoter region was inserted into the polyhedrin locus along with the egfp gene under the control of the p10 promoter (Fig. 11A). Since the vp1054 promoter and ORF sequences are overlapping with lef-10 ORF, the repair construct is also capable to express LEF-10. To investigate the function of the vp1054 gene, Sf9 cells were transfected with either the vp1054 knock-out or repair bacmid construct and monitored for EGFP expression by fluorescence microscopy. When Ac-vp1054 null construct was introduced into Sf9 cells, no viral propagation was observed in cell culture at 72 h to 120 h p.t. We could observe only a "single-cell infection" phenotype similar to the phenotype of Ac-gp64null bacmid (Fig. 11B). The results indicate that Ac-\(vp1054\)null is able to reach the very late phase of infection as confirmed by p10 promoter-driven EGFP expression. In other word, the results suggest that the expression of late expression factor 10, LEF-10, was not affected in \(vp1054\)-null bacmid mutant. From 72 h to 120 hours p.t., widespread EGFP expression could be seen in insect cell monolayers that were transfected with the repair constructs (Ac-\(\Delta vp1054\)-vp1054). The results are indicating that the repair bacmid is able to produce levels of infectious budded virions sufficient to initiate secondary infection at similar level as the wild-type bacmid (Fig. 11B). At 6 days p.t., cell culture supernatants were removed and added to freshly plated Sf9 cells and then incubated for 3 days to detect infection by virus generated from cells transfected with these bacmids. As expected, Sf9 cells incubated with supernatants from the transfections with the repair constructs showed numerous EGFP expressing cells (Fig. 11C). On the other hand, in insect cells incubated with supernatant from the transfection with the Ac-\(vp1054\)null knockout, no EGFP expression was detected at any time-point analyzed up to 72 h (Fig. 11C).

These results indicate that the vp1054 gene is essential for infectious BV production. It has clearly been proven that the 955-bp 3'-end sequence part of the vp1054 ORF can completely be deleted from the bacmid backbone and adequately rescued by introduction of the AcMNPV vp1054 ORF into a heterologous site (polyhedrin locus) of the genome. In addition, the results proved
that deletion of the \( vp1054 \) gene does not affect very late gene expression, as demonstrated by EGFP-positive cells in cells transfected with Ac-\( vp1054\)null bacmid mutant (Fig. 11B).

5 Generation and characterization of p6.9-null bacmid

To study the functionality of the AcMNPV p6.9 gene during virus infection, an vp80-null AcMNPV bacmid was constructed by deleting the p6.9 gene from AcMNPV bacmid (bMON14272) by homologous recombination in \( E. \) coli. The deletion construct was selected by its resistance to chloramphenicol that indicated that site-specific deletion of the p6.9 gene had occurred. In the resulting p6.9-null AcMNPV bacmid, the p6.9 gene was correctly replaced by the cat gene. Subsequently, the antibiotic resistance cassette (cat) was eliminated from bacmid backbone using \( Cre/LoxP \) recombination system (Fig. 12A). The deleted sequence was removed from the translational start codon (ATG→Met) to the stop codon (TAT→Tyr), nucleotide coordinates 86716 to 86879 according to the AcMNPV clone C6 genome sequence (SEQ ID NO:1). The stop codon of the p6.9 orf was not removed since its sequence is overlapping with the stop codon of flanked \( lef-5 \) orf. The structure of all the deletion constructs was confirmed by PCR (Fig. 12B). When the p6.9 gene is present, as in the parental wild-type AcMNPV bacmid, a 596-bp PCR fragment could be only amplified with cat-F and cat-R primers when cat gene was introduced into bacmid sequence in case of AcMNPV p6.9null(+cat) construct (Fig 12B). Correct recombination process was also confirmed by PCR mapping of p6.9 locus using primers 86596 and 86995. A 400-bp DNA fragment was amplified when wild-type AcMNPV bacmid was used as a template, whereas a 1220-bp DNA fragment could be amplified on AcMNPV i/p80-null(+caf) template (Fig. 12B). When final construct AcMNPV-vp80null(-caf) with eliminated cat cassette was used in PCR analysis, only a short 290-bp DNA fragment could be detected (Fig. 12B). Positive clones were successfully verified by DNA sequencing.

AcMNPV p6.9 gene is essential for viral replication

The repair constructs were designed such that the wild-type AcMNPV or SeMNPV p6.9 ORFs with AcMNPV p6.9 promoter region were inserted into the polyhedrin
locus along with the egfp gene under the p10 promoter (Fig. 13A). To investigate
the function of the p6.9 gene, Sf9 cells were transfected with either the p6.9
knock-out or repair bacmid constructs and monitored for EGFP expression by
fluorescence microscopy. When Ac-p6.9 null was introduced into Sf9 cells, no viral
propagation was observed in cell culture at 72 h to 120 h p.t. We could observe
only a "single-cell infection" phenotype similar to the phenotype of Ac-gp64null
bacmid (Fig. 13B). The results indicate that Ac-p6.9null is able to reach the very
late phase of infection as confirmed by p10 promoter-driven EGFP expression.
From 72 h to 120 hours p.t., widespread EGFP expression could be seen in insect
cell monolayers that were transfected with the two repair constructs (Ac-Δp6.9-
Acp6.9 and Ac-Δp6.9-Sep6.9). The results are indicating that these two repair
bacmids are able to produce levels of infectious budded virions sufficient to initiate
secondary infection at similar level as the wild-type bacmid (Fig. 13B). At 6 days
p.t., cell culture supernatants were removed and added to freshly plated Sf9 cells
and then incubated for 3 days to detect infection by virus generated from cells
transfected with these bacmids. As expected, Sf9 cells incubated with
supernatants from the transfections with the repair constructs showed numerous
EGFP expressing cells (Fig. 13C). On the other hand, in insect cells incubated
with supernatant from the transfection with the Ac-p6.9null knockout, no EGFP
expression was detected at any time-point analyzed up to 72 h (Fig. 3C).
Moreover, to characterize the exact effect of deletion of the p6.9 gene on AcMNPV
infection, the viral propagation in transfected Sf9 cells was compared between Ac-
wt, Ac-Δp6.9, Ac-Δp6.9-Acp6.9Rep, Ac-Δp6.9-Sep6.9Rep). Cell culture
supernatants of all the above bacmid constructs were analysed at indicated time
points for BV production (Fig. 13D). As expected, the repaired Ac-Δp6.9-
Acp6.9Rep and Ac-Δp6.9-Sep6.9Rep viruses showed kinetics of viral replication
consistent with wild-type virus (Ac-wt) propagation.

These results indicate that the p6.9 gene is essential for infectious BV production.
It has clearly been proven that the whole sequence of p6.9 ORF can completely
be deleted from the bacmid backbone and adequately rescued by introduction of
the AcMNPV vp80 ORF into a heterologous site (polyhedln locus) of the genome.
We also showed that p6.9 gene can be complemented efficiently by the SeMNPV-
derived p6.9 ORF (M. Westenberg). In addition, the results proved that deletion of the p6.9 gene does not affect very late gene expression, as demonstrated by EGFP-positive cells in cells transfected with Ac-p6.9null bacmid mutant (Fig. 15B).

Example II. The inventors have amended the best mode of the present invention in the following example.

Materials and Methods

Generation of an antibiotic resistance gene-free AcMNPV vp80-nul bacmid

To determine whether the VP80 protein has an essential role in the context of viral progeny production, we constructed an AcMNPV bacmid (derived from bMON14272 (from Invitrogen)) with a deletion of the vp80 ORF by homologous recombination in E. coli. To accomplish this, a cat gene flanked by mutant LoxP sites (Suzuki et al., 2005) was amplified using PCR primers vp80-KO-F and vp80-KO-R (see Table 1) from a plasmid comprising a cat gene flanked by mutant LoxP sites. The resulting PCR fragment, which contained the cat gene flanked by mutant LoxP sites and AcMNPV ~50-bp homology sequences to the 5’ or 3’ proximal region of the vp80 ORF, was treated with DpnI and gel-purified to eliminate the template plasmid. The PCR product was then transformed into DH1 0β E. coli cells containing bMON14272 (Invitrogen) and the Lambda RED recombinase-producing plasmid pKD46 (Datsenko & Wanner, 2000), which had been prepared in the following manner. Transformed DH1 0β-bMON14272/pKD46 E. coli cells were grown in 50-ml LB (2.0% peptone, 0.5% yeast extract, 85.5 mM NaCl, [pH 7.0]) cultures with kanamycin (50 µg/ml), ampicillin (100 µg/ml) and L-arabinose (1.5 mg/ml) at 30°C to an OD$_{600}$ of ~0.6 and then made electrocompetent by a standard procedure. The electroporated cells were incubated at 37°C for 3 h in 3 ml LB medium and plated on LB-agar containing chloramphenicol at a concentration of 6.5 µg/ml. After 48-h incubation at 37°C, the chloramphenicol-resistant colonies were streaked to fresh LB-agar medium with 34 µg/ml chloramphenicol. The plates were incubated at 37°C overnight, and colonies resistant to chloramphenicol were selected for further confirmation of the relevant genotype by PCR. Primers 90292 and 90889 were used to confirm the
absence of the vp80 ORF, and primers cat-F and cat-R were employed to verify the presence of cat cassette into bacmid (detailed sequences in Table 1).

To eliminate the introduced antibiotic resistance gene (cat) from the bacmid backbone, a Cre/LoxP recombinase system was employed. A Cre recombinase-carrying plasmid pCRE obtained from Jeanine Louwerse (LUMC Leiden, The Netherlands) was introduced into DH10b-bMON14272-vp80null E. coli cells, and CRE expression was subsequently induced by the addition of isopropyl thiogalactoside (IPTG). Briefly, the electroporated cells were incubated at 37°C for 3 h in 3 ml of LB medium (2.0% peptone, 0.5% yeast extract, 85.5 mM NaCl, [pH 7.0]) and plated on LB-agar medium containing 50 µg/ml kanamycin, 100 µg/ml ampicillin and 2mM IPTG. After 24-h incubation, colonies resistant to kanamycin and ampicillin were selected for further verification of the desired genotype by PCR. In PCR-based analysis, primers 89507 and 91713 (Table 1) were used to verify elimination of cat gene from bacmid backbone. Positive clones were also confirmed by DNA-sequencing.

To recover transposition competence, the helper transposase-encoding plasmid pMON7124 (Invitrogen) was re-introduced into DH10β-bMON14272-vp80null E. coli cells. Finally, the egfp reporter gene was introduced into the vp80-null bacmid to facilitate observation of its behaviour in insect cells. Briefly, the egfp reporter gene was amplified using PCR oligonucleotides gfp-N?el-F and gfp-Sp/?l-R (Table 1) from plasmid pEGFP-N3 (Clontech). The PCR product was cloned into plasmid pJet1.2/blunt using CloneJET™ PCR Cloning Kit (Fermentas) according to manufacturer’s protocol. Subsequently, the egfp ORF was excised from error-free pJet1.2-egfp with NheI and SphI and subcloned into Λf tel/Spftl-digested pFastBacDUAL (Invitrogen), to generate plasmid pFB-egfp. An expression cassette containing the egfp reporter gene under transcriptional control of the very late p10 promoter was transposed from pFB-egfp into polyhedhn locus of vp80-null bacmid as described in the Bac-to-Bac manual (Invitrogen). In the resulting genome, the complete vp80 ORF has been removed (see Figure 2). This corresponds to the deletion of 2074 bp from nucleotide positions 89564 to 91637 in the AcMNPV clone C6 genome provided in SEQ ID NO: 1.
Construction of repaired vp80-nu\bacmids

To prepare vp80 repair donor vectors, we modified plasmid pFB-egfp (noted above) by removing the polyhedrin promoter and replacing it with a fragment containing the vp80 promoter region and the vp80 ORF. First, a 2300-bp fragment containing both the vp80 promoter and ORF sequence was amplified using primers pvp80-Sful-F and vp80-Xbal-R (Table 1) from bacmid bMON14272 template, and cloned into vector pJetI .2/Blunt (Fermentas) to form pJetI .2-pvp80- vp80. After DNA sequence verification, the vp80 cassette was excised from pJetI .2-pvp80-vp80 by Stul/Xbal double digestion, and then subcloned into Bsf1 1071/ Xbal-digested and gel-purified pFB-egfp to generate donor plasmid pFB- egfp-pvp80-vp80. Parallely, a donor plasmid pFB-egfp-polh-vp80, where vp80 ORF is driven by the very late polyhedrin promoter (polh) was constructed. To this aim, a 2105-bp fragment carrying the vp80 ORF was amplified using primers vp80-Sacl-F and vp80-Xbal-R (Table 1) and cloned into pJetI .2/Blunt, to generate pJetI .2-vp80. In the final step, the vp80 ORF was cut out (SacI/XbaI) from pJetI .2-vp80, and subcloned into SacI/XbaI-digested pFB-egfp, to create pFB-egfp- polH-vp80.

To overcome a problem associated with the inavailability of anti-VP80 antibody, FLAG tag decoration (N- and C-terminus fusion) of VP80 was performed to facilitate immunodetection. The N-terminally fused FLAG-vp80 sequence was generated by a double-step PCR strategy, a so-called fusion PCR. First, a 259-bp fragment containing the vp80 promoter and the FLAG tag was PCR amplified using primers pvp80-Sful-F and vp80-FLAG-R1 from the bMON14272 bacmid template. After gel-purification and DNA quantification, the 259-bp fragment was used as forward primer in a second step PCR amplification with the reverse primer vp80- Xbal-R on the bMON14272 bacmid template. The final PCR product (2324 bp) was cloned into vector pJetI .2/Blunt (Fermentas) to form pJetI .2-pvp80-FLAG- vp80. After DNA sequence verification, the FLAG-vp80 cassette was excised from pJetI .2-pvp80-FLAG-vp80 by Stul/Xbal double digestion, and then subcloned into Bsf1 1071/ Xbal-digested and gel-purified pFB-egfp to generate donor plasmid pFB- egfp-pvp80-FLAG-vp80. The C-terminally fused vp80-FLAG cassette was amplified using pvp80-Sful-F and vp80-FLAG-R from the bMON14272 bacmid
template. The 2324-bp fragment was cloned into pJeti .2/Blunt, and subsequently transfer into pFB-egfp in a similar way as previous constructs.

The inserts of all developed donor plasmids were transposed into the vp80-null bacmid following the Bac-to-Bac protocol (Invitrogen). Screening of transposition-positive constructs into the polh locus was done by a the triplex PCR-based assay employing a M13 forward and reverse primers and a gentamicin resistance gene-specific primer GenR (Table 1).

Transfection-infection assay

Bacmid DNAs were prepared from 1.5-ml over-night bacterial cultures of 2 to 3 independent colonies carrying the bacmid with the inserted heterologous gene according to the Bac-to-Bac manual (Invitrogen) and were analyzed in parallel. For transfections, 1 µg of each bacmid DNA preparation was used to transfect 1x10⁶ Sf9 cells in a 6-well plate by the Cellfectin™-based transfection protocol as described in the Bac-to-Bac (Invitrogen) manual. From 72 h to 120 h post transfection (p.t.), viral propagation was checked by fluorescence microscopy. At 120 h p.t., the cell culture medium was centrifuged for 5 min at 2000 x g to remove cell debris, and this clarified supernatant was used to infect 1.5x10⁶ Sf9 cells in 6-well plates. After 72 h p.i., the spread of virus infection was again monitored by fluorescence microscopy. In all experiments, a wild-type bMON14272 bacmid carrying the egfp reporter gene under control of the p10 promoter was used as positive control. A bMON14272-gp64null bacmid also carrying the egfp reporter gene under control p10 promoter served as negative control, since it has lost the ability of cell-to-cell movement of the infection (Lung et al., 2002).

Time-course characterization of viral propagation in cell culture

Time course analyses were performed to compare budded virus production of the AcMNPV-vp δOnull virus and the various repair constructs in comparison to the wild type AcMNPV bacmid (Ac-wt) all containing egfp. Briefly, the Sf9 cells were seeded in 6-well tissue culture plates (1x10⁶ cells/well in 1 ml Sf900-II culture medium without serum at 28°C. After two hours, the culture medium was removed, and the cells were transfected with 5 µg bacmid DNA, under standard conditions as recommended in Bac-to-Bac manual (Invitrogen). Cell culture supernatants
were harvested at 24, 48, 72, 96 and 120 h p.t., and analysed for the production of infectious budded virus by an end-point dilution assay to determine the tissue culture infective dose 50 (TCID<sub>50</sub>). Infection was determined by monitoring egfp expression (from the p10 promoter). The average values of infectious titers derived from three independent transfections were calculated and plotted into graphs.

**Transmission electron microscopy**

Insect Sf9 cells were seeded in 25T flask (3.5x10<sup>6</sup> cells/flask), and transfected with 20 μg either the Ac-Δvp80, rescue Ac-Δvp80-VP80 or Ac-wt bacmid construct. After 48 h p.t., the cells were harvested and prepared for transmission electron microscopy as described previously (van Lent et al., 1990). Samples were examined and photographed with a Philips CM12 electron microscope.

**Budded virus production assay**

Insect Sf9 cells were seeded in two 25T flasks (3.5x10<sup>6</sup> cells/flask), and transfected with 20 μg either Ac-Avp80, Ac-Avp80-VP80, Ac-Avp80-pH-VP80, Ac-Avp80-FLAG-VP80, Ac-Avp80-VP80-FLAG, or Ac-wt bacmid construct. Five days p.t., the BV-enhched cell culture supernatants were harvested, and ultracentrifuged through a cushion of 10% sucrose solution (25,000 rpm for 1.5 hour, Beckman SW32). Pelleted budded virions were resuspended in sterile demi-water, and prepared for either negative staining electron microscopy, SDS-polyacrylamide electrophoresis, or PCR-based detection (as mentioned the above).

**Purification of ODVs and rod-shaped structures from infected cells**

The presence of ODVs and rod-like structures in infected/transfected insect cells was analyzed by electron microscopy (EM). For this purpose, insect cells were harvested 48 h p.i., lysed and the cell lysates were ultracentrifuged through a 40% sucrose cushion in TE (1 mM Tris-HCl pH 7.4, 0.1 mM EDTA) buffer (45,000 rpm for 1 hour, Beckman SW55). Pellets were resuspended in sterile demi-water and analyzed by negative staining EM as described previously (van Lent et al., 1990).

**Purification and fractionation of BV and ODV virions**
To produce BVs, 3.0x10^7 Sf9 cells were infected with Ac-Δvp80-Flag-vp80 or control Ac-wt virus at an MOI=1. Six days p.i., 72 ml of BV-enriched medium was collected and centrifuged at 1,500 x g for 10 min. The supernatant was then ultracentrifuged at 80,000 x g (Beckman SW28 rotor) for 60 min at 4°C. The BV pellet was resuspended in 350 µl 0.1 x TE buffer, and loaded onto a linear sucrose gradient (25 to 56% (w/v)), and ultracentrifuged at 80,000 x g (Beckman SW55 rotor) for 90 min at 4°C. The formed BV band was collected and diluted in 12 ml 0.1 x TE. The BV preparation was concentrated at 80,000 x g for 60 min at 4°C. The final virus pellet was resuspended in 150 µl of 0.1 x TE.

To produce ODVs, 6.0x10^7 Sf9 cells were co-infected with Ac-Δvp80-Flag-vp80 (MOI=25) and AcMNPV (MOI=5) viruses (strain E2, Smith & Summers, 1979). Five days p.i., the infected cells were harvested, and ODVs were purified from viral occlusion bodies as described previously (Braunagel et al., 1994). The final ODV pellet was resuspended in 0.5 ml of 0.1 x TE (10 mM Tris, 1 mM EDTA, pH=7.5). The purified BV and ODV virions were fractionated into envelope and nucleocapsid fractions as described previously (Braunagel et al., 1994). Final fractions were processed for SDS-PAGE and immunoblotted against either mouse monoclonal anti-Flag antibody (Stratagene), rabbit polyclonal anti-VP39 antiserum (kindly provided by Lorena Passarelli, Kansas State University, USA), rabbit polyclonal anti-GP64 antiserum (kindly provided by Hualin Wang and Feifei Yin, Wuhan Institute of Virology, China (Yin et al., 2008), or rabbit polyclonal antiserum against per os infectivity factor 1 (PIF-1) (kindly provided by Ke Peng, Wageningen University, The Netherlands (Peng et al., 2010).

Development of transgenic Sf9-derived cell line expressing vp80

To develop a cell line, which produces the VP80 protein, a 2105-bp fragment carrying the vp80 ORF was amplified using primers vp80-Sacl-F and vp80-Xba-R (Table 1) and cloned into pJeti .2/Blunt, to generate pJeti .2-vp80. In the next step, the vp80 ORF was cut out (SacI/XbaI) from pJeti .2-vp80, and subcloned into Sacl/XbaI-digested plZ (Invitrogen), to create plZ-vp80. The resulting plasmid vector plZ-vp80 was linearized with Eco57I, and gel-purified. Sf9 cells were seeded in six-well plate (1x10^6 cells/well), and transfected with 10 µg of the
linearized vector. After 24 hours post-transfection, cells were selected by cell culture medium containing Zeocin™ (300 µg/ml) for 2 to 3 weeks, until no control Sf9 cells survived under the same conditions. Cells were then propagated as an uncloned cell line.

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**Recombinant protein expression with the \textit{vp80nu|} virus**

To measure the capacity to express recombinant protein with the Ac-Δvp80 (trans-complemented) virus seed, 3.0x10^7 non-transformed Sf9 cells were infected (independent triplicate assay) with Ac-wt, Ac-Δvp80-Flag-vp80 (both produced in non-transformed cell line) or Ac-Δvp80 virus (produced in the Sf9-vp80 cell line) at a MOI=1.0. All of these virus seeds are expressing \textit{egfp} as a model heterologous gene from the baculovirus very late \textit{p10} promoter. At 48 h and 72 h p.i. cells and culture medium were harvested and used for Western blotting, enzyme-linked immunosorbent assay (ELISA) or BV titration (see above). For Western blotting the same antibodies as mentioned above were used to detect the Flag-tag, EGFP, and GP64, as well as a monoclonal mouse anti-actin antibody (ImmunO).

For relative quantification, Maxisorp 96-well plates (Nunc) were coated overnight at 4°C with 100 ng of rabbit polyclonal anti-GFP antibody (Molecular Probes) in a volume of 100 µl per well, which was followed standard ELISA procedures as previously described (Fric et al., 2008). The percentage of EGFP production was calculated (independent triplicate assay) according to the formula: \( \% \text{ EGFP expression} = \frac{(\text{test absorbance}_{n\text{h}} - \text{background absorbance})}{(\text{Ac-wt EGFP}_{72\text{h}} - \text{background absorbance}) \times 100\%}, \) where \( n\text{h} \) represents the time point p.i. The statistical significance of the observed differences between the control Ac-wt and the experimental Ac-Δvp80-Flag-vp80 and Ac-Δvp80 genotypes was analyzed with the Student's \( t \)-test.

**Results**

30
The AcMNPV vp80 gene is essential for viral replication

An AcMNPV deletion virus was constructed as detailed in Fig. 2. Repair constructs were designed such that the wild-type vp80 ORF or N- and C-terminally FLAG-tagged vp80 genes along with its native or polyhedrin promoter regions were inserted into the polyhedrin locus along with the egfp gene under the p10 promoter (Fig. 3A). To investigate the function of the vp80 gene, Sf9 cells were transfected with either the knock-out or repair bacmid constructs and monitored for EGFP expression by fluorescence microscopy. When Ac-vp80 null was introduced into Sf9 cells, no viral propagation was observed in cell culture at 72 h to 120 h p.t. We could observe only a "single-cell infection" phenotype similar to the phenotype of Ac-gp64null bacmid (Fig. 3B). The results indicate that Ac-vp80null is able to reach the very late phase of infection as confirmed by p10 promoter-driven EGFP expression. From 72 h to 120 hours p.t., widespread EGFP expression could be seen in insect cell monolayers that were transfected with the three repair (vp80 driven from its native promoter, vp80 driven from polyhedrin promoter and N-terminally FLAG-tagged vp80 driven from its native promoter) constructs indicating that these bacmids were able to produce levels of infectious budded virions sufficient to initiate secondary infection at similar level as the wild-type bacmid (Fig. 3B). In contrast, in insect cells transfected with C-terminally FLAG-tagged vp80 repair constructs, by 72 h p.t. EGFP expression was only observed in isolated cells that were initially transfected indicating that this bacmid construct is defective in viral replication (Fig. 3B). However, by 96 h p.t. formation of tiny plaques was observed and by 120 h p.t. very few plaques of normal size were developed. The results show that the C-terminal flagged mutant is strongly delayed in producing budded virus and showed that an unmodified C-terminus is very important for the function of VP80. At 5 days p.t., cell culture supernatants were removed and added to freshly plated Sf9 cells and then incubated for 3 days to detect infection by virus generated from cells transfected with these bacmids. As expected, Sf9 cells incubated with supernatants from the transfections with repair constructs showed numerous EGFP expressing cells (Fig. 3C). Nevertheless, cells incubated with supernatant from C-terminally FLAG-tagged constructs showed a significant reduction in the number of EGFP-positive cells. On the other hand, in insect cells incubated with supernatant from the transfection with the vp80
knockout, no EGFP expression was detected at any time-point analyzed up to 72 h (Fig. 3C).

Moreover, to characterize the exact effect of deletion of the vp80 gene on AcMNPV infection, the viral propagation in transfected Sf9 cells was compared between Ac-wt, Ac-Δvp80, Ac-Δvp80-vp80Rep, Ac-Δvp80-polh-vp80Rep, Ac-Δvp80-FLAG-vp80Rep and Ac-Δvp80-vp80-FLAGRep. Cell culture supernatants of all the above bacmid constructs were analysed at indicated time points for BV production (Fig. 4). As expected, the repaired Ac-Δvp80-vp80Rep, Ac-Δvp80-polh-vp80Rep, Ac-Δvp80-FLAG-vp80Rep viruses showed kinetics of viral replication consistent with wild-type virus (Ac-wt) propagation. Budded virion production by the C-terminal flagged Ac-Δvp80-vp80-FLAGRep virus was reduced to approximately 0.06% compared to the Ac-wt virus or the other repaired viruses.

These results indicate that the vp80 gene is essential for infectious BV production. It has clearly been proven that the whole sequence of vp80 ORF can completely be deleted from the bacmid backbone and adequately rescued by introduction of the vp80 ORF into a heterologous site (polyhedrin locus) of the genome. We also showed that vp80 gene expression can be driven by the heterologous polyhedrin promoter sequence with no negative effect on viral replication in cell culture. Additionally, we observed that the N-terminus in contrast to the C-terminus of VP80 is permissive to gene modifications (epitope tag-labeling). We noted that the kinetics of the C-terminally FLAG-tagged VP80 virus was significantly delayed when compared with all other rescue or wild-type viruses, indicating the functional importance of the VP80 C-terminus.

**VP80 is required for production of both BV and ODV**

The results described above indicated that the Ac-vp80null mutant is completely defective in production of infectious budded virus. However, there was also a possibility that the mutant can still produce non-infectious budded particles. To investigate the ability, Sf9 cells were transfected with either the knock-out, repair or wild-type bacmid constructs and 7 days p.t. cell culture mediums were ultracentrifuged to pellet budded viruses. The formed pellets were either analyzed
by negative staining electron microscopy or by Western blot- and PCR-based
detection to confirm the presence of the budded viruses. No intact budded virus,
virus-like particles, nor its structures (such as major capsid protein VP39 and viral
genome sequence) were revealed in the pellet from the cells transfected with the
Ac-i/p80null mutant (Fig. 5A and 5B). On the other hand, all analyzed repair
constructs produced normally-appearing budded virus as compared with budded
virus-derived from the wild-type virus (Fig. 5A). Nevertheless, it was very difficult to
find representative budded virions in the pellet derived from C-terminally FLAG-
tagged vp80 gene repair construct-transfected cells.

To further characterize deletion of the vp80 gene on baculovirus life cycle, electron
microscopy was performed with ultra-thin sections generated from bacmid-
transfected cells. The Ac- vp80null-transfected cells developed typically phenotype
of baculovirus-infected cell with an enlarged nucleus, a fragmented host
chromatin, an electron-dense virogenic stroma, etc. (Fig. 6A). The absence of
VP80 did not prevent formation of normally-appearing nucleocapsids inside the
virogenic stroma (Fig. 6C). The formed nucleocapsids were phenotypically
undistinguishable from those produced by either the Ac-i/p80null repair or Ac-wt
bacmids. However, an abundance of assembled nucleocapsids was rather less as
compared with cells-transfected with the Ac-i/p80null repair or Ac-wt bacmids (Fig.
6E and 6G). In addition, no occlusion-derived virions nor bundles of nucleocapsids
prior to an envelopment could be observed in the peristromal compartment of a
nucleoplasm (so called the ring zone) of Ac- vp80null bacmid-transfected cells (Fig.
6B and 6D). It seems that VP80 plays a role during maturation of nucleocapsids
and/or their release/transport from the virogenic stroma. Eventually, VP80 can
somehow contribute to an efficient nucleocapsid assembly which could be
explained by small number of nucleocapsids present in the virogenic stroma of Ac-
vp80null transfected cells. When the vp80 gene was re-introduced back into the
bacmid mutant, a lot of nucleocapsids and occlusion-derived virions could be seen
in the ring zones of transfected cells (Fig. 6F). An abundance and morphology of
occlusion-derived virions produced in Ac-Δvp80-vp80 repair bacmid-transfected
cells were similar to those produced by wild-type bacmid (Fig. 6F and 6H).
VP80 is associated with nucleocapsids of both BV and ODV

To investigate the association of VP80 with BV preparations, BVs were collected at 48 h p.i. and nucleocapsid and envelope fractions were separated. The Flag-VP80 protein was only detected in the nucleocapsid fraction as a double-band of molecular masses ranging between 80-kDa and 95-kDa that were observed in infected Sf9 cells (Fig. 14A, upper panel). Correct separation into nucleocapsid and envelope fractions was confirmed with antibodies against VP39 (nucleocapsid only) and GP64 (envelope only) (Fig. 14A, lower panels).

To examine whether VP80 is also associated with ODVs, Sf9 cells were co-infected with the Ac-Δvp80-Flag-vp80 and occlusion body (OB)-producing wt AcMNPV viruses to provide the POLH protein. Western blot analysis showed that VP80 associates with the nucleocapsid fraction of ODVs and in this case migrates as a single band of ~80 kDa, corresponding to the 80-kDa form produced in the very late phase of infection (Fig. 14B, upper panel). Proper fractionation into nucleocapsid and envelope fractions was controlled with antiserum against PIF-1, an ODV envelope protein (Fig. 14B, lower panel).

The function of VP80 can be rescued by genetic trans-complementation

To verify whether a vp80 deletion in the viral genome can be complemented by a vp80 ORF offered in trans under control of a constitutive promoter, a transgenic cell line expressing Flag-tagged vp80 was constructed. In these cells VP80 was mainly produced as a protein of approximately 95-kDa as was shown by Western blot analysis with anti-Flag antibody (Fig. 15A). Two minor bands, one of ~80-kDa and second of ~65-kDa were also observed.

In trans-complementation assays, Sf9-vp80 cells were transfected with the Ac-Avp80 bacmid, and the spread of virus infection was monitored by EGFP-specific fluorescence at 96 h and 120 h p.t. (Fig. 15Ba-c). Viral plaques were seen in the transfected Sf9-vp80 cells demonstrating that the virus was transmitted from cell to cell. Nevertheless, we noted that the number and size of the developed plaques was significantly smaller than observed in Sf9 cells transfected with the Ac-wt
bacmid (Fig. 15Bd). As a control, non-transgenic Sf9 cells showed only single-cell infections when transfected with the Ac-Δvp80 bacmid (Fig. 15Bc).

When the culture medium of the Ac-Δvp80 transfected Sf9-vp80 cells was used to infect freshly seeded non-transgenic Sf9 cells a "single-cell infection" phenotype was observed (Fig. 15Bb, right panel). Hence, the BV particles resulting from trans-complementation were able to enter cells but were defective in producing new BV. This also shows that the Ac-Δvp80 did not revert to Ac-wt in the Sf9-vp80 cells, by picking up the transgene from the host cells. As predicted, no EGFP signal was detected in Sf9 cells receiving the supernatant from Ac-Δvp80-transfected, non-transgenic Sf9 cells (Fig. 15Bc, right panel). The numbers of infectious BVs released from the Sf9-vp80 cells transfected with the Ac-Δvp80 bacmid were compared with those produced in Sf9 cells transfected with Ac-wt at 6 days p.i. This experiment showed that the current trans-complementation system is approximately 25 fold less effective in BV production than the classical Sf9-based production system (Fig. 15C).

Trans-complemented, replication-deficient Ac-vp80null virus is competent to express high levels of recombinant protein

To assess the effect of the vp80 gene deletion on the level of recombinant protein expression, a bench-scale comparative production assay has been performed. Herein, the Sf9 cells were in parallel infected with three types of baculovirus seeds at an MOI=10, namely (i) Ac-wt, (ii) Ac-Δvp80-Flag vp80 (both produced in Sf9 cells), and (iii) Ac-Δvp80 (produced in Sf9-vp80 cells) all encoding EGFP. Western blotting profiles showed that the EGFP protein was expressed at identical levels for all three tested baculovirus genotypes as was the GP64 glycoprotein which served here for control purposes (Fig. 16A, upper panel). The relative amount of EGFP was quantified by ELISA at 48 and 72 h p.i in infected cell lysates (Fig. 16B) and did not reveal any statistically significant difference in EGFP levels between the three tested baculovirus genotypes. The results thus demonstrate that the trans-complemented Ac-Δvp80 virus seed, although defective in viral replication, is as capable to produce recombinant protein as conventional baculovirus.
expression vectors as long as the initial multiplicity of infection is high enough to infect all cells.

Also during the production culture, revertant virus genotypes carrying the *vp80* gene were not detected, as no *de novo* expressed Flag-VPβO protein (Fig. 16A) was detected in immunoblots. Theoretically, a certain quantity of Flag-VPβO protein associated with the trans-complemented virus seed is entering the insect cells, but this was no longer detected at very late times post-infection and is probably degraded by either lysosome- or proteasome-mediated activity. In the same experiment, no BV release was recorded in cell culture supernatants originated from Sf9 cells inoculated with the Ac-Δvp80 virus seed (Fig. 16C), demonstrating that neither revertant virus generation nor wild-type virus contamination had occurred.

**Summary**

In this study we focused on the improvement of conventional baculovirus-based expression tools with the goal to eliminate contaminating baculovirus progeny from manufactured recombinant protein(s). This effort is strongly driven by pharmaceutical perspectives, since recombinant baculovirus-expressed therapeutics are being more and more used in human and veterinary medicine. Hence, we aimed to identify baculovirus gene(s), whose targeting results in a deficiency of baculovirus virion production, but does not or, only mildly affect very late gene expression. In this way high level expression of heterologous genes will be safeguarded.

A summarizing overview of the new technology with the *vp80* gene as example is presented in Fig. 17. Using bacmid-based engineering the inventors constructed an AcMNPV genome lacking the *vp80* gene (Fig. 17B). Functional genomics and electron microscopy analyses revealed that *vp80* deficiency prevents production of both BVs and ODVs. In parallel, Sf9 cells were engineered to produce VP80 to trans-complement the Ac-Δvp80 knock-out bacmid (Fig. 17A.C). Finally, we proved that frans-complemented, replication-deficient baculovirus seed is capable
to produce an amount of recombinant protein similar to that produced by conventional baculovirus vectors.

Table 1. List of PCR primers in order of appearance in the text.

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<th>SEQ ID #</th>
<th>Primer name</th>
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multicapsid nucleopolyhedrovirus: Sequencing, transcription and


1. A method for the production of a biopharmaceutical product, comprising:
   (a) infecting a biopharmaceutical-producing insect cell with at least one baculovirus, said at least one baculovirus comprising a genome coding for said biopharmaceutical product, and
   (b) maintaining the biopharmaceutical-producing insect cell under conditions such that the biopharmaceutical product is produced, wherein the genome of said at least one baculovirus is deficient for at least one gene essential for proper baculovirus virion assembly or wherein said biopharmaceutical-producing insect cell comprises an expression control system allowing the inactivation of at least one gene essential for proper baculovirus virion assembly.

2. The method according to claim 1, wherein said at least one gene essential for proper baculovirus virion assembly is made deficient in said genome by way of nucleotide substitution, insertion or deletion.

3. The method according to claim 1, wherein the biopharmaceutical-producing insect cell is a recombinant insect cell comprising a construct expressing a dsRNA specific for the at least one gene essential for proper baculovirus virion assembly, the dsRNA being optionally expressed under an inducible promoter.

4. The method according to any one of claims 1 to 3, wherein the at least one baculovirus is produced before step (a) in a baculovirus-producing cell expressing a complementing copy of the at least one gene essential for proper baculovirus virion assembly.

5. The method according to any one of claims 1 to 4, wherein the at least one gene essential for proper baculovirus virion assembly is selected from vp80, vp39, vp1 054 and p6.9.
6. The method according to any one of claims 1 to 5, wherein the deficiency or inactivation of the at least one gene essential for proper baculovirus virion assembly does not affect very late expression from said baculovirus in comparison to very late expression from wild-type baculovirus.

7. The method according to any one of claims 1 to 6, wherein the at least one baculovirus is derived from AcMNPV or BmNPV.

8. The method according to any one of claims 1 to 7, wherein the biopharmaceutical product is a recombinant protein, a recombinant virus or a virus-like particle.

9. The method according to claim 8, wherein the biopharmaceutical product is a recombinant AAV.

10. The method according to any one of claims 1 to 9, wherein the biopharmaceutical product is coded by at least one gene introduced in the recombinant baculovirus genome under the control of the polyhedrin or p10 promoter.

11. Use of a baculovirus-insect cell system for the production of a biopharmaceutical product wherein the baculovirus-insect cell system comprises a baculovirus-producing insect cell infected with at least one recombinant baculovirus, wherein:

- the, or each, recombinant baculovirus comprises a baculoviral genome that encodes the biopharmaceutical product, or at least one component of the biopharmaceutical product, and

- the, or each, recombinant baculoviral genome is deficient for at least one gene essential for proper assembly of said baculovirus, or the baculovirus-producing insect cell comprises an expression control system allowing the inactivation of the at least one gene essential for proper baculovirus virion assembly.
12. A bacmid comprising a baculoviral genome, wherein said genome is deficient for a gene essential for proper baculovirus virion assembly, preferably wherein the genome of said baculovirus is deficient for vp80, vp39, vp1054 or p6.9.

13. A recombinant baculovirus vector, preferably an AcMNPV baculovirus vector, wherein the genome of said baculovirus is deficient for a gene essential for proper baculovirus virion assembly, preferably wherein the genome of said baculovirus is deficient for vp80, vp39, vp1054 or p6.9.


15. An insect cell, comprising a construct expressing a dsRNA specific of a gene essential for proper baculovirus virion assembly, preferably directed against vp80, vp39, vp1054 or p6.9, said construct being preferably integrated in the genome of the insect cell.

16. An insect cell comprising an expression cassette coding for a gene essential for proper baculovirus virion assembly.

17. The insect cell according to claim 16, wherein said gene is vp80, vp39, vp1054 and/or p6.9.

18. A method for the production of a baculovirus deficient for at least one gene essential for proper baculovirus virion assembly, comprising the step of transfecting an insect cell according to claim 16 or 17 with a bacmid according to claim 12, wherein the gene essential for proper baculovirus virion assembly for which said bacmid is deficient is the gene encoded by the expression cassette comprised in said insect cell.

19. A method for screening baculoviral genes the inactivation of which could be useful for producing biopharmaceuticals without contaminating baculovirus virions in an insect cell - baculovirus system, comprising:
   a) providing a cell culture of cells containing a baculoviral genome;
b) contacting said cell culture with means for inactivating at least one test baculoviral gene of said baculoviral genome, for example with RNA interference; and
c) testing virion formation from said cell culture in comparison to virion formation from a cell culture not contacted with said means;
wherein a test gene is selected as potentially useful for producing biopharmaceuticals if its inactivation results in a reduction of baculoviral virion formation.

20. The method according to claim 19, further comprising step d) of testing very late gene expression from the cell culture contacted with said means in comparison to very late gene expression from a cell culture not contacted with said means;
wherein a test gene is selected as potentially useful for producing biopharmaceuticals if its inactivation results in a reduction of baculoviral virion formation and if it does not affect very late gene expression from said baculoviral genome.
Fig 1
Fig 2
Fig 4
Fig 5
Fig 9
Fig 11
Fig 12
Fig 13
Fig. 15
Fig. 16
Fig. 17