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Review

Baculovirus expression vector system: An efficient tool for the production of heterologous recombinant proteins

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Today, the recombinant proteins become a major foundation of different industrial sectors. Products of these industries enhance the fields of medicine, pharmacology, agriculture, nutrition, petrochemicals, paper, textiles and many others. Since the mid 1980s, recombinant DNA technology has enabled the development of a wide diversity of vectors for expression of the desired recombinant protein in numerous prokaryotic and eukaryotic organisms. Baculovirus expression vector system is considered one of the most successful and widely acceptable means for the production of recombinant proteins in extremely large quantities. Proper posttranslational modifications of the expressed proteins in insect cells, the usual host of baculoviruses, get them soluble, correctly folded and biologically active products. Along with the convenient biological safety, the easy scale up production of recombinant proteins into industrial levels brings this system the first choice in most cases. In the current review, different aspects of the baculovirus biology, structure and morphology are briefly covered to establish the basic concepts important for understanding the mechanisms involved in utilization of baculoviruses as expression vectors. Few outlines on the baculovirus expression system are discussed in terms of potentials, limitations, strategies and perspectives.

Key words: Baculovirus, expression, recombinant, protein.

BASICS OF BACULOVIROLOGY

Insect viruses have been of interest and concern to humans for a long time, as first indicated by scientific reports and literatures during the 16th and 17th centuries (Friesen and Miller, 2001). Intensive studies in the 19th and 20th centuries lead to the isolation of many insect viruses and gathering valuable information regarding their structure and replication. Isolation of baculoviruses, in particular, considered a landmark event in studying these viruses, besides it opened avenues for utilization of insect viruses in molecular biology (Bergold, 1947).

Baculoviruses are the most prominent viruses known to infect the insect population. They have been reported in more than 600 different insect species, particularly those of the orders *Lepidoptera*, *Hymenoptera*, *Diptera*, *Coleoptera*, *Neuptera*, *Siphonoptera* and *Trichptera*, as well as in the crustacean order *Decapoda* (shrimp) (Couch, 1974). The name baculovirus was originated from the Latin word *baculum*, meaning “stick” which refers to the morphology of the virus nucleocapsid (Fauquet et al., 2005). Although, baculoviruses infect a wide diverse of arthropod species, individual isolates normally show a limited host range and infect only closely related species. Therefore, baculoviruses are often named according to the primary species which they infect and isolated from (Miller and Lu, 1997).

Abbreviations: OBs, Occlusion bodies; **NPV,** *nucleopolyhedrovirus*; **GV,** *granulovirus*; **SNPV,** single nucleopolyhedrovirus; **MNPV,** multiple nucleopolyhedrovirus; **BV,** budded virions; **OV,** occluded virions; **ORF,** open reading frame; **PABP,** polyhedrin promoter-binding protein; **BEVS,** baculovirus expression vector system.

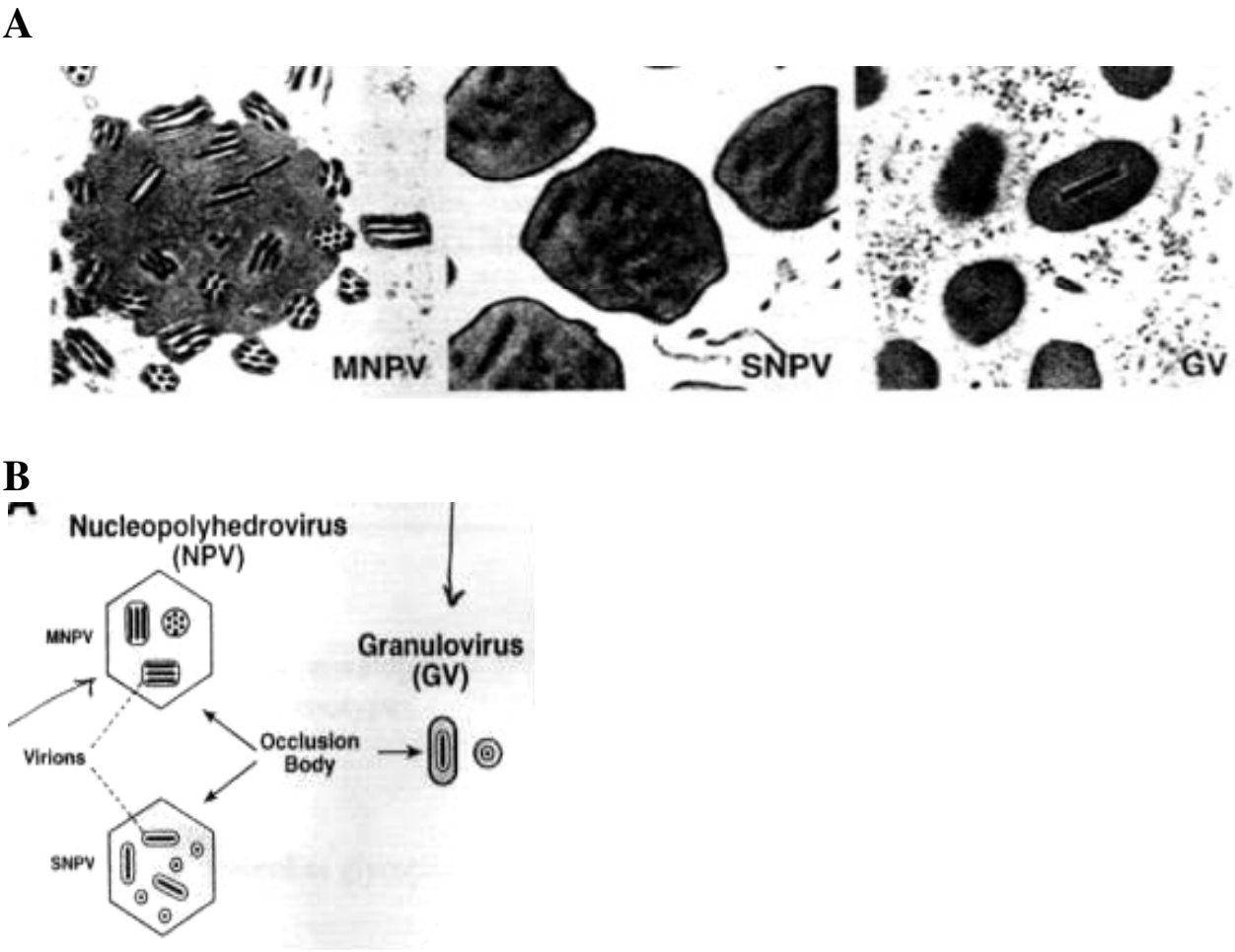

Figure 1. Occlusion bodies of SNPV, MNPV and GV. A, Electron microscopy of cells infected with SNPV, MNPV and GV, respectively; B, scheme of occlusion bodies of SNPV, MNPV and GV. [Adapted from Rohrmann, G.F. (1992): Baculovirus structural proteins. J. Gen. Virol., 73: 749-761].

The family *Baculoviridae*: Organization aspects

Members of the family *Baculoviridae* are characterized by deposition of crystalline proteineous structures in the nuclei of infected cells during late stages of infection. These structures are termed occlusion bodies (OBs) and found to be responsible for protection of baculoviruses from the environmental inactivating factors such as dissection, UV radiation and nucleases; thus, allowing them to remain viable for long periods in nature (Slack and Arif, 2007). According to the shape, size, structure and the major protein that forms the OBs, family *Baculoviridae* was classified into two main genera: *nucleopolyhedrovirus* (NPV) and *granulovirus* (GV) (Figure 1) (Friesen and Miller, 2001). Viruses belonging to the genus *Granulovirus* have small ovicylindrical OBs of 0.25 to 0.5 µm in diameter that contain single virions and compose of a protein with granular appearance under light microscope called granulin (Tanada and Hess, 1991). By contrary, the members of genus *nucleopolyhedrovirus* have polyhedral OBs, which are much larger in diameter (1 to 15 µm) and composed of a closely related protein called polyhedrin. NPVs can be separated further according to the number of encapsulated nucleocapsids within a common envelope. Some viruses contain just one nucleocapsid per envelope (single nucleopolyhedrosis viruses; SNPVs), while others contain many (3 to 10) nucleocapsids per envelope (multiple nucleopolyhedrosis viruses; MNPVs) (Rohrmann, 2008).

Baculovirus structure and assembly

The baculoviruses have two morphologically distinct phenotypes which are sequentially produced during the replication cycle; (1) budded virions (BV); (2) occluded virions (OV) (Figure 2). The basic structure of both phenotypes is the rod shaped nucleocapid, which is 250 to 300 nm long and 30 to 60 nm in diameter (Funk et al.,

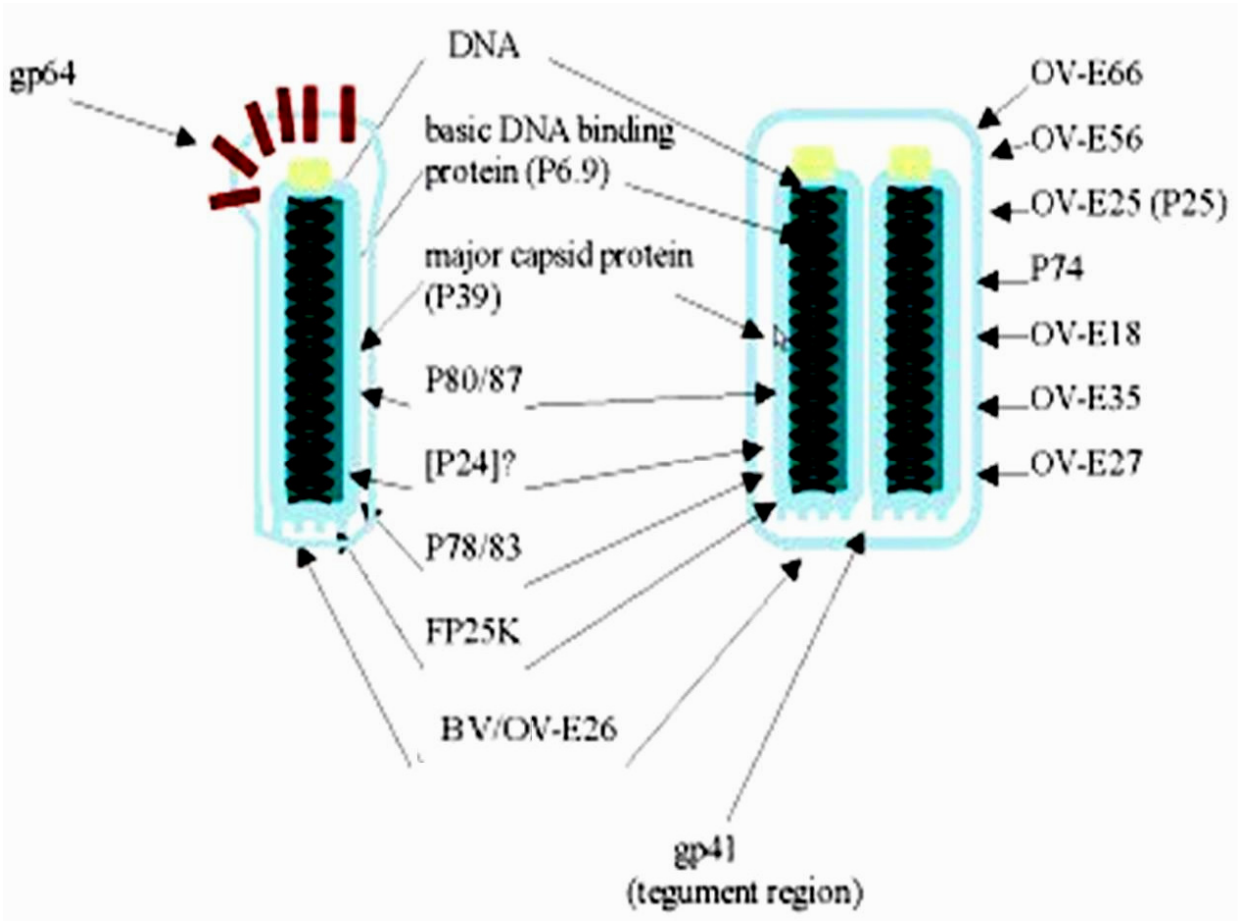

Figure 2. Scheme of occlusion bodies of SNPV and MNPV. [Adapted from Van Oers, M.M. and Vlak, J.M. (2007). Baculovirus genomics. Curr. Drug Targets].

1997). The nucleocapsid contains a single molecule of circular, supercoiled double-stranded DNA genome, which is tightly associated with a small protein called P6.9. This protein is rich in the arginine amino acid and closely related to the protamines, a class of proteins present in many fish, avian and mammalian sperm nuclei and involved in the production of highly condensed DNA molecules (Wilson et al., 1987). In addition, several other proteins complete the nucleocapsid structure including; the major capsid protein (P39), the minor capsid protein (P24) and a proline rich phosphoprotein (P78/83), beside the ill characterized protein (P87). Both of P39 and P24 are randomly distributed over the surface of the nucleocapsid, while P78/83 is localized only at the terminal pole. The precise location of the fourth protein (P87) has not been established yet (Rohrmann, 2008).

After complete intranuclear assembly of the viral nucleocapsids, they are directed to form one or another of the virus phenotypes. The nucleocapsids which are destined to become BV pass through the nuclear membranes and acquire a temporary envelope carrying the virus coded protein P16. This membrane is completely lost at the time the virus reaches the plasma membrane. During virus budding from the plasma membrane, it acquires another loose-fitting envelope that carries terminal peplomers of the major virus-specific protein gp64/67. Such protein plays an essential role for initiating infection of other insect host cells (Monsma et al., 1996).

On the other hand, the nucleocapsids destined to become OV remain in the nucleus and acquire a *de novo* envelope from unknown origin. Single or multiple nucleocapsids are packaged within a single envelope, depending on the type of the virus involved, which in turn embedded in a crystalline matrix consists of a single protein, either polyhedrin or granulin, to form OBs. Surrounding the matrix of OBs, an electron dense envelope termed calyx or polyhedron envelope (PE) is formed for further protection of OV (Braunagel and Summers, 2007). This envelope was originally reported to be composed from carbohydrates only (Minion et al., 1979), however, a phosphoprotein component termed PE protein or pp34 has been subsequently identified (Whitt and Manning, 1988). Several other proteins associated with OV but not with BV were identified, nevertheless, their exact role is not well known. Three of which P25, P74 and gp41, appears to be associated with the polyhedral envelope, while a fourth (P10) is not clear whether it

constitutes an essential component of OBs or it simply trapped during the occlusion process (Russell and Rohrmann, 1993).

The biphasic life cycle of baculoviruses

The complexity of baculovirus life cycle appears to be in part as a result of the presence of two viral phenotypes formed in different stages of the viral replication, with each phenotype having a specific role during the life cycle (Rohrmann, 2008). OV is the initial form responsible for primary infection of insect larvae during oral ingestion of plants contaminated with OBs. The midgut juices of larvae are highly alkaline (pH 9 to 12) and provide a suitable environment for dissolving OB membranes for the release of their embedded virions (Kelly, 1982). Liberated viruses infect the midgut columnar epithelial cells by a process of receptor-mediated fusion. After the transfer of nucleocapsid to the nucleus of infected cells and uncoating of the DNA genome, a normal sequence of replication events occurs including formation of virogenic stroma, nucleocapsid synthesis and envelopment. Secondary infection of other host insect cells is predominately achieved by BV produced in the midgut cells. Spread of BV occurs through the haemolymph, the epithelial cells lining the tracheal network or both (Keddie et al., 1989). Nearly all internal tissues of the insect are affected especially hypodermis, fat bodies, muscles, ganglia and pericardial cells. In these tissues, particularly fat bodies, massive quantities of OBs are accumulated in the nucleus, reaching 10^8 OBs per larva or 10% of the larval dry weight. Finally, the larva is converted into a milky white liquid in a process called “melting” and the huge number of OBs is disseminated in the nature to begin another cycle of larval infection (Slack and Arif, 2007).

Baculovirus genome expression and regulation

The baculovirus genome is a large covalently closed, supercoiled, circular DNA of 80 to 220 kbp in size. Genomic DNA is infectious and does not require the existence of virion-associated proteins for replication after cellular entry and uncoating. To date, the complete genomic sequence of at least 48 baculoviruses has been reported and the sequence data showed the presence of 337 ORFs encoding for more than 100 structural and non structural proteins (Fauquet et al., 2005; Van Oers and Vlak, 2007; GenBank, 2010). The structural components of the virus and OBs account for at most 30 genes only (Ayres et al., 1994), while the rest is responsible for encoding proteins required for replication process and assembly (Tolmalski et al., 1988). The ORFs are closely spaced on both DNA strands and are separated by intergenic regions having a high A-T content, which constitute the promoters and termination sequences for the individual viral genes (Possee and Rohrmann, 1997).

The general strategy of gene expression and regulation in baculoviruses is nearly similar to other large animal DNA viruses like pox and herpes. Gene expression is regulated in a cascade-like fashion, in which activation of each class of genes relies on the synthesis of proteins from the previous gene classes. This temporal regulation allowed organization of baculovirus genes into three main classes: early (E), late (L) and very late (VL) (Friesen and Miller, 2001). Many literatures suggested subdivision of early genes into two classes: immediate early (IE) and delayed early genes into two classes: immediate early (IE) and delayed early (DE), in which some IE proteins are required for expression of DE genes (Fuarino and Summers, 1986; Theilmann and Steward, 1993). The early genes do not require any viral proteins for their activation and are transcribed during the first 4 h post infection with the aid of host cell RNA polymerase II. E genes' protein products have several regulatory functions essential for transcription of L and VL genes and replication of the viral DNA (Huh and Weaver, 1990). Moreover, these products block cell cycle progression leading to accumulation of infected cells in the G2/M phases (Ikeda and Kobayoski, 1999). L genes are transcribed after 12 to 24 h of infection in the presence of viral α-amantidine resistant RNA polymerase II and results in the synthesis of new genomic DNA copies and production of the structural proteins of budded and occluded virions. VL genes (polyhedrin and P10) are overexpressed during late stages of infection (18 to 76 h pi) to produce the proteins essential for encapsulation of OV and formation of OBs (Braunagel and Summers, 2007).

The polyhedrin

Polyhedrin is the major protein component of the polyhedra (NPV OBs). It is encoded by a single copy gene of approximately 735 bp, with no introns found to interrupt its sequence (Van Oers and Vlak, 2007). The protein has a remarkably stable size of 243 to 246 aa and a molecular mass of about 29 kDa. The entire DNA sequence of the polyhedrin gene was completely identified for different baculovirus strains and showed a high degree of nucleotide sequence identity that exceed 80%. Thus, it represents the most conserved baculovirus gene known until now. Polyhedrin has demonstrated two specialized functions which are important for baculovirus structure and multiplication; first, it forms a protective layer around the virus against adverse environmental conditions and second, it resists solubilization except under strongly alkaline conditions of the insect mid gut, therefore, it promotes the initiation of primary infection of the insect larvae (Rohrmann, 2008).

The regulatory mechanisms controlling synthesis of the

polyhedrin have taken a considerable attention because such protein is produced in massive quantities in late stages of viral infection. It was found that up to 18% or more of the total proteins in baculovirus infected cells were polyhedrin. Several mechanisms were suggested to explain the overexpression nature of the polyhedrin gene, among which the strong polyhedrin promoter is the leading factor (Luckow and Summers, 1988). The most striking feature of this promoter is a highly conserved sequence of 8 nucleotides (TAAGTATT) at the transcription start point (Rankin et al., 1988). Several other viral genes showed to have *trans*-regulating activities on the polyhedrin region (Li et al., 1993). In addition, a cellular factor, the polyhedrin promoter-binding protein (PABP), was identified, purified and found to bind with high specificity and affinity to transcriptionally important motifs of the polyhedrin promoter (Burma et al., 1994). Polyhedrin transcription is also enhanced by a homologous region sequence (*hr1*) located 3.7 kb upstream of the polyhedron promoter (Habib et al., 1996).

Although, a hyper-expressed gene product, polyhedrin gene is not necessary for viral replication in cell culture since virus growth in culture is basically depend on BV phenotype, with no need to the OV form. This was exploited in the development of the baculovirus expression system, in which the polyhedrin gene is removed and replaced by a foreign gene under the control of the potential polyhedrin promoter and other transcriptional activating factors (Smith et al., 1983; Pennock et al., 1984).

BACULOVIRUSES AS EXPRESSION VECTORS: CONCEPTS AND STRATEGIES

Over the last decades, baculoviruses have been emerged and established among the most popular gene expression systems. They are distinguished by their capacity to produce unusually high levels of biologically active foreign recombinant proteins (Hunt, 2005). The pioneering work that led to the development of this system was carried out at Max Summers laboratory in Texas A and M, USA (Smith et al., 1983). Since this time, when the first protein interleukin 2 (IL-2) was produced, the system has been invested massively to express a broad range of important prokaryotic and eukaryotic proteins (more than 1000 recorded protein). Consequential modifications have been introduced continuously to the system and reflected in its efficacy, versatility and popularity (Galleno and Sick, 1999).

The baculovirus expression vector system (BEVS) offers many desirable features that are undoubtedly responsible for its popularity. The key elements of this success lie principally in its efficiency in expressing high levels of recombinant proteins that may exceed 600 µg/ml and the ease of scale up production (Luckow et al., 1993; Abdulrahman et al., 2009). Since BEVS is based on expression in eukaryotic (insect) cells, it permits proper protein folding, oligomerization and post-translational modifications such as proteolysis, N- and O-linked glycosylation, phosphorylation, palmitolation, myristolation, fatty acid acylation and amidation, in a manner that is nearly resembling those occurring in mammalian cells (Kato et al., 2010). Consequently, the expressed recombinant proteins are often soluble, correctly folded and biologically active; thereby, they provide valuable products for the use in vaccine development, therapeutic, diagnostic purposes and structure/function studies (Jarvis, 2009). In addition, baculoviruses are known to infect arthropods only with a narrow host range. Although, these viruses may enter certain types of mammalian, fish and plant cells, they are not infectious to them (Friesen and Miller, 2001). Therefore, BEVS possess little or even no safety problems either during preparation of the recombinant proteins or during their use in practice.

Despite these potential advantages, particular patterns of posttranslational processing must be empirically determined for each virus construct (Frank, 2005). Proteins expressed in insect and mammalian cells are not exactly the same. Fundamental differences in the glycoprotein processing pathways of insects and higher eukaryotes have been described (Marchal et al., 2001). These differences lead to the production of a less complex and trimmed glycoproteins with no sialic acid side chains. Such glycoproteins are varying significantly from their authentic forms and are mostly inactive (Bienkowska-Szewczyk and Szewczyk, 1999). Absence of sialic acid termini in particular, constitutes a major problem because these terminal sugars often have a direct or indirect influence on the glycoprotein function and antigenicity (Jenkis and Curling, 1999). Novel baculovirus vectors and insect cell lines have been designed in the last few years to overcome these problems and to enable scientists to produce glycoproteins that resemble their authentic forms (Jarvis et al., 2001; Palomares et al., 2003). Another drawback of this system is that baculovirus genes are known to lack introns and as a result the use of genomic DNA clones is not recommended since proper splicing of heterologous genes is doubtful. Although, few reports demonstrated the ability of insect cells to splice complex genes, the use of cDNA clones is still the ideal (Jeang et al., 1987).

Utilization of baculoviruses as expression vectors appears to be basically depending on two main features. First, the virus contains a number of genes that are usually expressed late in infection and are not essential for the virus replication in tissue culture. Replacement of one or another of these genes with the foreign genes does not affect the growth of recombinant viruses in cell culture. Second, many of these genes are present under the control of powerful promoters and regulatory elements, which allow abundant expression of the heterologous gene products (Smith et al., 1983; Weyer et al., 1990). Most of the available baculovirus expression systems nowadays utilize polyhedrin or P10 promoters

with their associated flanking regions (Hunt, 2005). Nevertheless, other promoters like those of P6.9, P39 and P35 genes appear as efficient as polyhedrin and P10, but their use is still limited and not recommended (Bonning et al., 1994).

Originally, the manipulation of baculoviruses was tedious and time-consuming because their genomes are too large and few restriction sites are available for the insertion of foreign genes. However, some reports described the direct insertion of pieces of DNA into the baculovirus genome through enzymatic ligation or through the use of transposable elements (Peakman et al., 1992; Luckow et al., 1993). Currently, the usual way to construct recombinant baculoviruses is by introduction of the desired foreign gene into a transfer (shuttle) vector, which is an ordinary bacterial plasmid carrying portions of the baculovirus genome spanning the gene promoter and transcription termination used for gene expression. Subsequently, the foreign gene is introduced in the baculovirus genome by co-transfecting insect cells with the infectious viral DNA and the transfer vector. Recombination between homologous sequences in the viral DNA and the transfer vector results in insertion of the heterologous gene within the virus genome and generation of recombinant viruses (Lopez-Ferber et al., 1995).

Traditional baculovirus expresses systems based on a low frequency recombination (0.1 to 2%) between the shuttle vector and the circular viral genome. This frequency was improved (up to 50%) by linearization of the baculovirus DNA with a unique restriction site close to the target site for insertion. Linearized virus DNA has reduced infectivity and cannot initiate a viral infection in most cases unless rescued by the recombination event, which is responsible for recircularization of the viral genome (Summers and Smith, 1988). New strategies have been developed recently to raise the recombination efficiency over 90% and to permit better identification of recombinant viruses (Miao et al., 2006).

Several valuable modifications to the baculovirus expression system are continuously accumulated and represent a potential impetus to its efficacy, speed and simplicity. For example, the use of liposome-mediated transfection of insect cells increased the transfection efficiency and consequently, the titer of generated recombinant virus stock (Hartig et al., 1991). Moreover, the development of a wide range of transfer vectors carrying lac-Z gene gave the option to simply identify recombinant viruses' colormetrically (Rohrman, 2008).

CONCLUSION

Baculovirus expression vectors continue to prove competence and gain new fields of practice day after day. A huge range of proteins from human, animal, plant, bacterial, viral, fungal and protozoan were expressed by such technology and were utilized in the development of new vaccines, diagnostics and therapeutic agents. Future advancement of technology and application of the BEVS is an expected issue that promises a better exploitation of this technology in biology and medicine.

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