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MOLECULAR AND APPLIED ASPECTS OF VIROLOGY

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Baculovirus Expression Systems for Production of Recombinant Proteins in Insect and Mammalian Cells

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Abstract—Baculovirus vector systems are extensively used for the expression of foreign gene products in insect and mammalian cells. New advances increase the possibilities and applications of the baculovirus expression system, which makes it possible to express multiple genes simultaneously within a single infected insect cell and to obtain multimeric proteins functionally similar to their natural analogs. Recombinant viruses with expression cassettes active in mammalian cells are used to deliver and express genes in mammalian cells in vitro and in vivo. Further improvement of the baculovirus expression system and its adaptation to specific target cells can open up a wide variety of applications. The review considers recent achievements in the use of modified baculoviruses to express recombinant proteins in eukaryotic cells, advantages and drawbacks of the baculovirus expression system, and ways to optimize the expression of recombinant proteins in both insect and mammalian cell lines.

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INTRODUCTION

Studies are continuously performed to improve both vectors used to transfer foreign DNA into mammalian cells and eukaryotic expression systems that are based on such vectors and allow efficient production of biologically active proteins in vitro and in vivo. Various methods are used to deliver foreign genes into eukaryotic cells, including both physicochemical (electroporation, bombardment with gold or wolfram microparticles, etc.) and biological (lipid conjugates in the form of liposomes, recombinant viruses, etc.) techniques. Many vectors based on various viruses were developed in the past two decades to deliver foreign genes to target cells. Since viruses are natural carriers that transfer foreign DNA into mammalian cells, they help genetic material to enter the target cell and ensure a high rate of cell transfection both in vitro and in vivo and a high level of foreign gene expression.

Expression vectors based on baculoviruses make it possible to produce functional eukaryotic proteins in cultured insect cells with a yield higher than with any other eukaryotic heterologous gene expression system. Insect baculoviruses are capable of transferring genetic information into mammalian cells, and, consequently, baculovirus-based vectors can be used as expression vectors for mammalian cells. Since baculoviruses are not replicated when transferring genetic material in mammalian cells, baculovirus vectors are convenient and safe both in vitro and in vivo. Owing to their high capacity and biological safety, baculovirus vectors and their improvement attract particular attention. Baculovirus expression systems, which make it possible to efficiently produce functional eukaryotic proteins in a wide range of mammalian cells in vitro, ex vivo, and in vivo, provide a promising alternative to vectors based on human viruses. This review considers the specifics of baculoviruses and baculovirus expression systems.

VIRUS VECTORS FOR GENE TRANSFER AND EXPRESSION IN EUKARYOTIC CELLS

Vectors based on mammalian viruses, which are efficient in transferring genes into mammalian cells, are convenient as natural vectors to deliver foreign DNA to eukaryotic cells. The choice of an optimal system depends on many criteria. It is important to consider how simple the relevant technique is, how strong the promoters are, whether their temporal regulation is possible, how quick the target eukaryotic protein is produced in substantial amounts, etc. One or another vector is chosen depending on the particular problem.

Unfortunately, all of the known vector systems used to deliver reporter genes in vitro and in vivo are far from perfect, although the problem of delivering exogenous DNA in vitro is generally solved. Systems designed to deliver foreign genes into mammalian target cells in vivo are improved continuously. For instance, several characteristics of vector systems—

their stability in the integrated state, regulated expression, and safety—still need improvement. In the ideal case, not only the foreign gene should be stable but it should also be expressed efficiently for a long time.

Various vectors based on human viruses are mostly used to improve the systems intended for efficient delivery and expression of transgenes in eukaryotic cells in vitro and in vivo. Vectors are constructed on the basis of retroviruses, adenoviruses, adeno-associated viruses, herpes virus, lentiviruses, and baculoviruses. Each of the resulting systems has its advantages and drawbacks.

The baculovirus expression system is the most convenient, and its advantages are obvious. For instance, the transgene to be introduced may reach 100 kb, and the level of its expression and the production of the recombinant protein are high. A vector is suitable for simultaneous expression of several genes, including nonspliced ones. The virus is not replicated in mammalian cells and, consequently, can be used to deliver reporter genes into mammalian cells both in vitro and in vivo. The vector allows direct addressed cloning and both transient and stable transduction of mammalian cells. Various primary cells can be used for transduction with a broad range of cell specificities. The baculovirus is capable of infecting nondividing cells, lacks cytotoxicity, and is not infectious in humans. This is especially convenient because innate immunity to the majority of human viruses limits the use of the vectors based on these viruses. Baculoviruses provide a means to overcome this difficulty; i.e., recombinant viruses of a nonhuman origin can be constructed to serve as vectors for gene transfer. Humans lack both antibody- and cell-mediated innate immune memory against baculoviruses because baculoviruses are only infectious for insects.

The drawbacks of the baculovirus system is that posttranslational modification of proteins in insect cells differs from that in human cells in some cases and that baculoviruses are rather sensitive to the complement system when they are used to deliver foreign genes to mammalian cells and tissues in vivo. Notwithstanding, the baculovirus vector system is successfully used to deliver genetic information to eukaryotic cells in vitro, and its use to deliver genes in vivo is expanding. Vectors based on baculoviruses find increasing application in basic and applied biology, biomedicine, and development of new methods for diagnosis, vaccination, and gene therapy.

BACULOVIRUSES

Biology of Baculoviruses

What are baculoviruses (family Baculoviridae)? Early relevant data dating back to the 16th century describes "wilting disease" of silkworm, which, as is clear now, was caused by a baculovirus. Baculoviruses are pathogenic for arthropods, especially insects, infecting more than 30 insect species. The baculoviruses studied most comprehensively are nucleopolyhedrosis viruses (NPVs), in particular, *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV).

AcMNPV has a rodlike nucleocapsid, which is (35–40) × (200–400) nm in dimensions and is surrounded by a lipid envelope. The nucleocapsid contains circular double-stranded DNA (134 kb) with 154 open reading frames (ORFs). DNA is surrounded by the core protein p39 and major envelope protein gp64. The full-length genomic DNA sequence is known [1]. Replication and transcription of the virus genome take place in the host cell nucleus. DNA is packaged into a rodlike nucleocapsid in the nucleus as well [2, 3]. The infection process yields two forms of the virus, that is, two different types of virus particles. First, individual budded virions (BVs) form and mediate a horizontal spreading of infection through the cell population. At the last step of infection, individual virions interact to produce protein inclusion bodies, which are known as occlusion-derived virions (ODVs), range from 2 to 15 μm in dimension [4, 5], and consist of many virions (nucleocapsids) surrounded by a membrane. The baculovirus life cycle is shown in Fig. 1.

Molecular Biology of Baculoviruses

Baculoviruses have a unique two-phase infection cycle. The first phase is lytic; it is more infectious and is characterized by nucleocapsid production. The second, occlusion phase involves the production of polyhedrons, which contain polyhedrin as a major component [6]. Entering the infected cell, virus DNA gives origin to four generations of mRNAs and four generations of virus proteins. Each generation includes proteins that activate synthesis of the next generation. The main strategy regulating these processes is production of multiple overlapping mRNAs during four different time phases [7, 8]. An immediate early phase involves the expression of the genes that are involved in the initiation of virus DNA replication, the virus activator genes, and the genes whose transcription does not require virus activators. This group includes the genes for the IE1, IEN (IE2), and IEO proteins. A delayed early phase is characterized by the expression of the genes whose protein products are involved in virus genome replication. In this phase, IEO, IE1, and PE38 regulate transcription of the genes that are necessary for virus DNA replication. Proteins whose functions are necessary in early infection are synthesized at the early and middle phases. At the late phase, cell DNA replication is completed and both virus and host RNA polymerases transcribe the genes for virus structural proteins, which form nucleocapsids. The polyhedrin and P10 genes are transcribed at the very late phase.

Baculoviruses utilize the host transcription machinery and RNA polymerase II and their own enhancers (hrs) and transcriptional activators IEO,

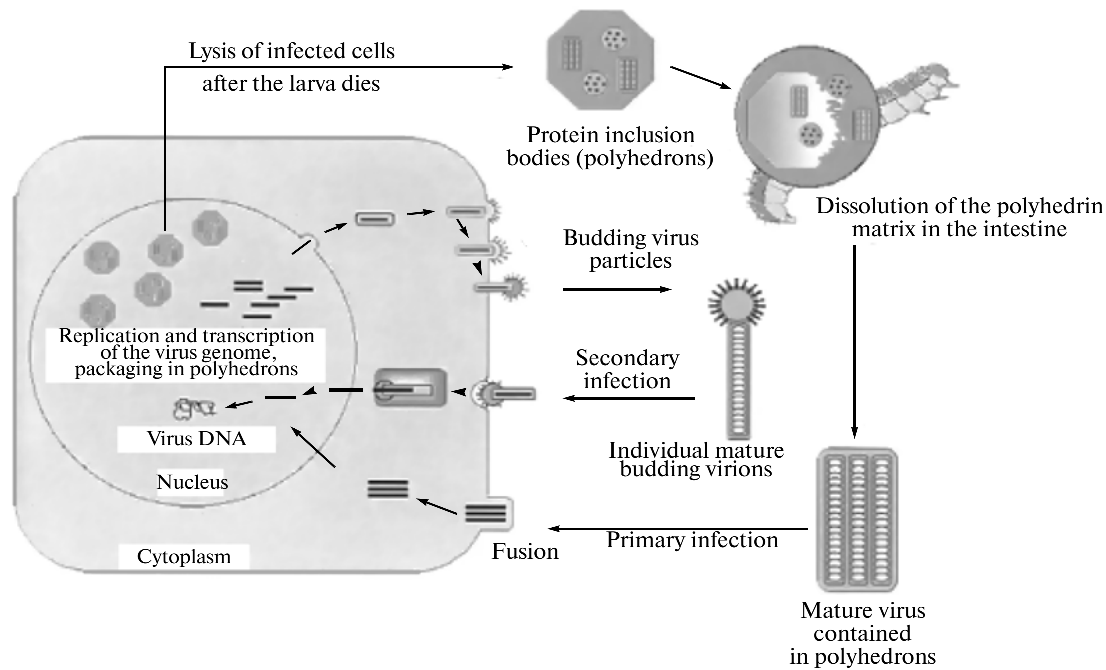

Fig. 1. Baculovirus life cycle. Infecting cells, the virus comes into the intestine of insect larvae with food and enters intestinal cells via endocytosis. Virus DNA is released from the capsid and enters the nucleus, where virus DNA is replicated and transcribed and a nucleocapsid forms. Extracellular intermediate virus particles bud from the plasma membrane of infected cells, being known as budded virions. At the last step of infection, individual budded virions are packaged to produce protein inclusion bodies, or polyhedrons, where virions efficiently survive in the environment.

IE1, and PE38 for transcription of their genes. It is clear that host RNA polymerase II transcribes the early genes of the virus. Both virus and host proteins are possibly involved in replication of the baculovirus genome in the cell nucleus, as is the case with other viruses. The mechanism of baculovirus reproduction, in particular, the mechanism of baculovirus genome replication in infected cells, and the general scheme of baculovirus DNA replication are still far from completely understood [8–10].

The main fourth-generation proteins, polyhedrin (24–29 kDa in different virus strains) and p10 (10 kDa), are produced in large amounts, accounting for 25–50% of total cell protein. Both of these proteins are involved in polyhedron formation and are not essential for virus replication. The polyhedrin and p10 genes have strong promoters, which ensure the production of the virus proteins in large amounts in late infection. The promoters of these genes are regulated by five regulatory elements, which are regularly distributed along virus DNA. Apart from polyhedrin and p10, the set of late proteins includes two other proteins that are not essential for replication. These are the protamine-like protein p6.9, which forms the nucleocapsid core, and the major capsid protein p39. It is at this stage that foreign gene sequences can be inserted under the control of the promoters of the late proteins. This stage provides a basis for construction of baculovirus expression vectors.

BACULOVIRUS EXPRESSION SYSTEMS FOR PRODUCTION OF RECOMBINANT PROTEINS IN EUKARYOTIC CELLS

Systems of Baculovirus Expression Vectors

A baculovirus expression system (BES) for producing recombinant proteins in insect cells came to be intensely studied and used in the early 1980s, when its application to express foreign proteins in *Spodoptera frugiperda* cells was reported [11–15]. A recombinant virus was used to infect insect cells as its natural host. During infection, the recombinant baculovirus is replicated, and the foreign proteins are synthesized. The potent promoter of the virus polyhedrin gene initiates transcription of the coding sequence at a high rate, ensuring a high yield of the protein. Since the virus life cycle is independent of the polyhedrin gene, its replacement with a foreign protein-coding gene in a recombinant baculovirus leads to the production of the heterologous protein in large amounts with post-translational modification similar or identical to that in mammalian cells. The protocol initially used to construct recombinant baculoviruses was modified and improved. In early studies, recombinant baculoviruses were identified visually by selecting the virus plaques with a polyhedrin-negative phenotype, whose frequency is usually 0.1–1.0%. The process was labor consuming and low efficient. The following techniques were developed more recently with insect cells.

One utilizes homologous recombination between a transport vector, which contains a reporter gene, and the polyhedrin locus of virus DNA to produce a recombinant virus. Site-specific recombination is utilized in a second technique, while a third one relies on site-specific transposition.

Vector DNAs should contain a virus DNA sequence of up to 7 kb to allow homologous recombination, a promoter, the 5' leader mRNA sequence of one of the baculovirus genes, an initiator codon, and all regulatory elements essential for the function of an expression vector. Smith and Summers [16–19] were the first to develop and patent the method whereby foreign genes are introduced into the baculovirus genome via homologous recombination between a transport vector containing a target gene sequence, which is to replace the polyhedrin gene, and virus DNA. The recombinant virus was identified visually under a microscope by the absence of polyhedrons in virus plaques. The recombination rate was no more than 2% with this method. The efficiency of recombination was improved more recently. First, circular virus DNA was linearized to limit the infection potential of the virus genome; then, *Escherichia coli lac*Z was introduced into the virus genome, and the *Bsu*361 unique restriction site was added to ORF603 of the polyhedrin locus. The recombination rate increased indeed to approximately 30%, but the recombinant virus had to be purified from the parental wild-type virus via several rounds of the plaque assay [20, 21].

The next step towards more efficient generation of recombinant baculoviruses with the use of a homologous recombination system was a development of a new technique, a flash BAC system, which obviated the need of plaque-assay purification and, thus, reduced the time it took to obtain high-titer preparations of a recombinant baculovirus. In this expression system, virus DNA has deletions from ORF1629, which is essential for baculovirus replication. The deletions inactivate the virus and prevent its replication in insect cells. At the same time, virus DNA contains a bacterial artificial chromosome (BAC) in place of the polyhedrin gene in the polyhedrin locus and, as a result, is capable of replication in a circular form in *E. coli* cells. Homologous recombination between modified virus DNA and vector DNA, which contains a target gene, restores the function of the gene essential for baculovirus replication and removes the BAC sequence. The recombinant baculovirus is replicated to yield a genetically homogeneous virus preparation. The system makes it possible to use various baculovirus vectors suitable for homologous recombination and improves the yield of secreted and membrane-associated proteins [22, 23].

A homologous recombination system based on site-specific recombination is another new technique. This is a combination of the GateWay system, which takes advantage of the fact that bacteriophage λ can be integrated into the *E. coli* chromosome via site-specific recombination in vitro, and the BaculoDirect system. A target gene is cloned between two recombination sites, attL1 and attL2, in an entry clone vector. Donor linear DNA contains these sites in the polyhedrin locus. LR recombination yields recombinant baculovirus DNA with the target gene inserted in the polyhedrin locus. Since linear DNA is used, production of nonrecombinant viruses is less likely, and the method yields the pure recombinant virus without an admixture of the parental wild-type virus (Fig. 2) [24, 25].

Finally, another method that has received wide acceptance is the Bac-to-Bac system, which allows all gene-engineering manipulations to obtain a recombinant expression vector in *E. coli* cells and involves site-specific transposition of the reporter gene and subsequent transfection of insect cells. The method is based on transposition of the target gene, which is flanked by the Tn7L and Tn7R elements of the Tn7 transposon, from a donor plasmid to a target plasmid (bacmid) at the *mini-att-Tn7* sites. Transposition is achieved using a helper plasmid, which carries the *tns-A-E* transposase gene. The bacmid contains the baculovirus genome, the *mini-F* replicon for stable replication in *E. coli* cells, and the *mini-att-Tn7* site inserted into *lac*Z. The method is convenient because bacterial cells are used to construct and identify baculovirus DNA [26–28].

The production of eukaryotic proteins in cultured insect cells is high and is still unachievable with any other eukaryotic heterologous gene expression system (up to 500 mg/l in most cases and up to 1–10 mg/l in the case of secreted proteins). The baculovirus genome is capable of accepting large foreign DNA segments. The upper limit of a foreign DNA sequence suitable for insertion into the baculovirus genome is still unknown. Recombinant baculoviruses with any additional DNA sequence propagate to a titer similar to that of the wild-type virus within the same period of time. DNA sequences remain unchanged and stable through many cell passages, and the level of gene expression does not decrease. One more advantage is that several genes may be expressed simultaneously in one infected insect cell to obtain multimeric proteins functionally similar to their natural analogs. In the majority of cases, the protein product remains soluble in insect cells, in contrast to proteins produced in bacteria. Recombinant proteins are localized in the same subcellular compartments as the corresponding native proteins. Nuclear recombinant proteins occur in the cell nucleus, membrane proteins are anchored in the cell membrane, and secreted proteins are involved in the cell secretory pathway. The foreign protein product commonly passes through all maturation and modification stages characteristic of eukaryotic systems, which is important for its full biological activity. Baculoviruses are not pathogenic to mammals or plants and are not infectious for mammalian cells, thus allowing both transient and stable transduction of mammalian cells.

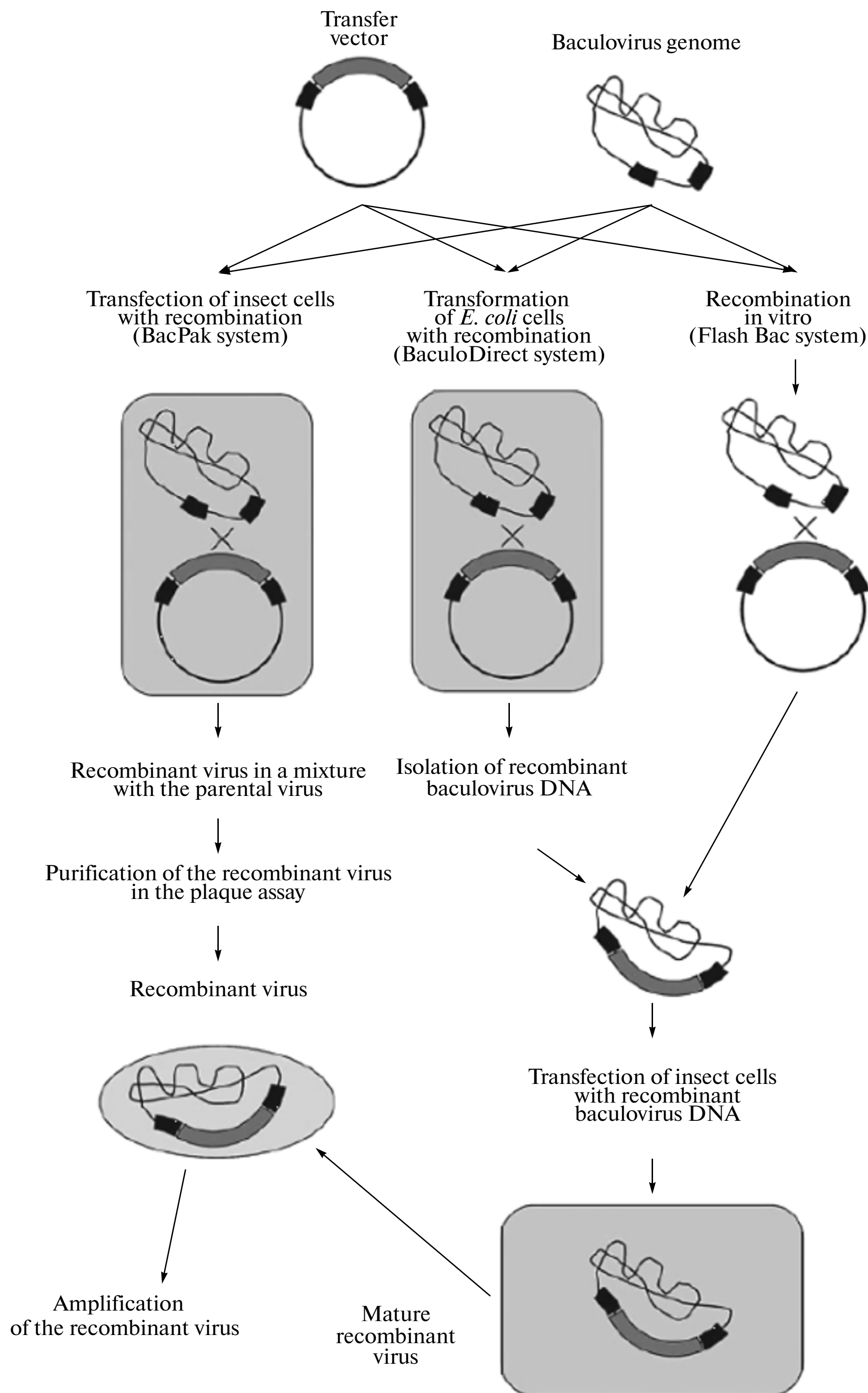

Fig. 2. Construction of a recombinant baculovirus for subsequent expression of a recombinant protein via homologous recombination between a transfer vector and the virus genome.

Generation of Functionally Active Recombinant Proteins in Insect Cells with the Use of the Baculovirus Expression System

In insect cells, proteins are processed through all stages of their maturation and modification characteristic of eukaryotic systems, including glycosylation, phosphorylation, palmitylation (acylation with fatty acid residues), amidation, carboxymethylation, signal peptide cleavage, and proteolytic cleavage. Recombinant proteins form disulfide bonds and assume neces-

sary secondary and tertiary structures. The sites of the above modifications usually coincide with their analogs occurring in the natural protein, and the recombinant protein is structurally and functionally similar to its natural counterpart. Production of eukaryotic proteins in the baculovirus expression systems mostly yields biologically active products retaining their antigenic properties.

The HIV surface antigen gp160 produced in the baculovirus system binds with monoclonal antibodies against authentic gp160 and with the CD4 receptor. The antigenic proteins of the recombinant gp160 do not differ from those of the natural gp160 [29]. The baculovirus expression system in insect cells made it possible to obtain a biologically active secreted form of the CD4 receptor for HIV-1 [30] and the α and β subunits of human horionic gonadotropin (HG) with the antigenic determinants of α-HG and β-HG [31]. The baculovirus expression system was used to produce the human P2 Kirsten-Ras(4B) protein [32]; human lymphocytic protein tyrosine kinase $p56^{lsk}$ [33]; correctly processed Ras type x1caax-1 [34]; and recombinant mouse entactin with a correctly processed signal peptide, which interacted with anti-entactin polyclonal antibodies and ensured cell attachment and aggregation in a culture, thus displaying the properties of the corresponding natural component of the matrix [35]. Belzhelarskaya and Satton [36] expressed the gene for the functional mouse serotonin receptor (5HT1) in cultured insect cells and showed that the recombinant 5HT1 possessed functional activity of the native receptor in the cell response to serotonin. Binding with the ligand with the help of the GTP-binding protein, the receptor activates phospholipase C to initiate a calcium flux in the cell. Grischuk et al. [37, 38] synthesized human steroid 21-hydroxylase (CYP21A2) and its new mutant variants in insect cells and studied the effects of the mutations of its gene on enzymatic activity. Hasemann and Capras [39] expressed the mouse immunoglobulin genes in insect cells. A recombinant vector plasmid contained the cDNAs of the heavy (H) and light (L) immunoglobulin chains. Both of the chains proved to be processed and glycosylated correctly and formed the H_2L_2 complex in insect cells. Ernst et al. [40] demonstrated the possibility of using the baculovirus system to construct and screen eukaryotic expression libraries. In total, more than 1000 various heterologous proteins were synthesized using the baculovirus system, and more than 95% of them underwent correct posttranslational modification.

However, N-glycosylation in insect cells does not always proceed the same way as in mammalian cells. Although protein glycosylation is incorrect to a certain extent, the final products have the same antigenic properties (by immunofluorescence analysis) as their authentic counterparts, as was demonstrated with the example of influenza virus hemagglutinin and neuraminidase [41]. It is possible that peak glycoprotein synthesis occurs earlier than peak synthesis of other proteins in infected cells as determined with respect to polyhedrin synthesis. The baculovirus early promoters make it possible to overcome the problem of inadequate glycosylation in some cases [42]. In addition, the cell capability of modifying proteins may be altered upon overexpression of foreign proteins [43]. In mammalian cells, N-glycosylation of glycoprotein polypeptide chains by glycosyltransferases yields protein products with N-glycans that have sialic acid and galactose as terminal residues. Insect cells seem to lack the corresponding glycosylases, and protein N-glycosylation is consequently incomplete in some cases [44–46] (Fig. 3).

To overcome these difficulties, Sf9 insect cells were genetically modified to construct a cell line (Mimic Sf9 insect cells, Invitrogen) that has the genes for α-2,6-sialyltransferase and β-1,4-galactosyltransferase. Infected with a recombinant baculovirus, cells of the line express the foreign gene to produce a functionally active protein with posttranslational modification characteristic of mammalian cells [47, 48]. Various approaches are used to increase the production of correctly processed functional proteins in insect cells infected with recombinant baculoviruses. For instance, the bovine β-1,4-galactosyltransferase gene was expressed in *Sf9* cells under the control of the baculovirus *ie1* early promoter. The resulting cells were infected with a recombinant baculovirus that carried a DNA sequence coding for the tissue plasminogen activator, and the final product proved to be galactosylated [49, 50]. Baculovirus early promoters help to solve the problem of inadequate posttranslational modification of recombinant proteins in insect cells in late infection, when enhanced synthesis of a foreign protein impairs the cell capability of modifying the protein product. New transgenic *Sf9* cell lines were constructed, each demonstrating more efficient N-glycosylation as compared with original *Spodoptera frugiperda* cells and producing recombinant glycoproteins with mammalian N-glycans [50–53].

The production of the functional secreted form of the lutropin receptor ectodomain with adequate N-glycosylation increases when its gene is controlled by the *P10* promoter (compared with the polyhedrin *PH* promoter) [53]. The expression of functional polydnavirus vankyrin is more efficient when its gene is controlled by baculovirus *P10* compared with *PH* [54]. Kulakovskii et al. [55] studied the extent of N-glycosylation of secreted placental alkaline phosphatase synthesized in *Spodoptera frugiperda* and *Trichoplusia ni* cell lines infected with recombinant baculoviruses. The extent of oligosaccharide fucosylation in *S. frugiperda* cells was higher than in any of the *T. ni* cells under study [55]. Attempts are continuously made to further improve the baculovirus expression system to synthesize human-like glycoproteins in insect cells [45, 47, 57].

Another drawback of the baculovirus expression system is that the processing of heterologous proteins

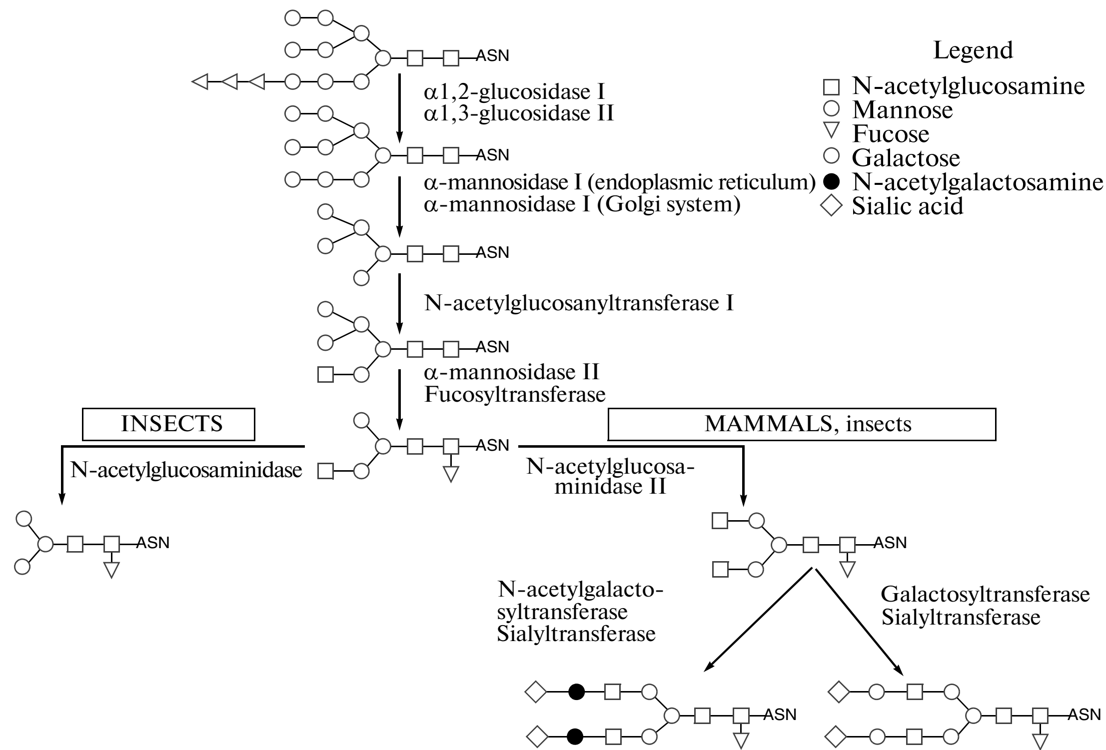

Fig. 3. Protein N-glycosylation in insect and mammalian cells.

is inefficient when the proteins are synthesized as large inactive protein precursors, such as peptide hormones, neuropeptides, growth factors, metalloproteases, and certain other proteins. In some cases, when a native protein functions as a heterodimer or has tissue- or species-specific modification, the analog synthesized in the baculovirus system is functionally inactive unless its binding partner (a receptor or a ligand) or modifying enzyme is coexpressed in the same system [58]. Insect cells are unable to fully process a large amount of protein precursors probably because the content of the furin-like enzyme is not high enough [59–61]. Posthouse et al. [62] showed that furin coexpression increases convertase activity in the baculovirus expression system.

Secretion of heterologous IgG in insect cells infected with a recombinant baculovirus results in insoluble immunoglobulin. However, when the HSP70 chaperone is coexpressed together with immunoglobulin, soluble intracellular immunoglobulin is produced, and the amount of the secreted protein increases [63]. In some cases, when a protein forms a multimeric structure via disulfide bridging of identical subunits, its expression in a biologically active multimeric form is possible. For instance, the baculovirus expression system was used to produce VP60, a unique capsid component of the rabbit hemorrhagic disease virus (RHDV). When the VP60 cDNA was cloned under the control of the polyhedrin promoter, the recombinant protein formed not only correct dimers of 80 kDa but also aberrant dimers of 100 and 62 kDa, which were a result of a translational frameshift. The dimers formed via disulfide bridging [64].

Since recombinant proteins can undergo proteolytic cleavage in the baculovirus expression system, it is possible to produce a precursor protein that is then cleaved by cell protease to yield several different polypeptides. Expression of the HIV-1 precursor protein (55 kDa) from the polyhedrin gene promoter makes it possible to obtain three different structural proteins of HIV-1 [65]. A biologically active human interleukin 1β-converting enzyme (HICE) was also obtained. The biologically active enzyme is a heterodimer consisting of 10- and 20-kDa polypeptides. The cDNA for the HICE precursor was expressed under the control of the polyhedrin promoter. After synthesis, the polypeptide chain of the precursor was cleaved at the Asp116–Asp117 bond, and the polypeptide chains formed the HICE heterodimer. The cleavage was determined by the protein itself, namely, by its proteolytic (pro) region [66].

In some cases, it is necessary to express the cDNAs for individual subunits separately in order to obtain a multimeric protein. One of the means to achieve this is to cotransfect cultured insect cells with two or more recombinant DNAs containing the subunit genes. A mature active product is not always obtained in such experiments because the assembly of a multisubunit

complex in insect cells may substantially differ from that in natural conditions. An attempt was made to employ this method in producing influenza virus polymerase, which consists of three subunits [67]. Each of the three polymerase genes— *pb1*, *bp2*, and *bpA*—displayed good expression from the polyhedrin promoter. Infection of insect cells with PB1 and PB2 recombinant virus DNA yielded a heterodimer of the corresponding subunits. However, when cells were cotransfected with all three recombinant DNAs, a complex of the three subunits was not produced. In is possible that insect cells failed to ensure specific modification of PBA or that correct assembly of the complex required additional genes of the influenza virus.

Generation of Virus-like Particles and Vaccines in Insect Cells with the Use of the Baculovirus Expression System

The baculovirus expression system in insect cells makes it possible to study the assembly of virus-like particles in the absence of an infectious virus and is often use to develop candidate vaccines based on virus-like particles with usual recombinant antigens [68–71].

Expression cassettes with three, four, or more promoters of the virus late genes are constructed to express the genes for complex proteins, proteins consisting of a few subunits, and multisubunit complexes in insect cells. For instance, virus-like particles with four recombinant virus proteins were obtained for the bluetongue virus (BTV), whose virion contains seven structural proteins. For this purpose, the pAcAB4 transfer vector, which contained a block of BTV gene sequences (*VP2*, *VP6*, *VP7* and *NS1*) under the control of two *pH* promoters and two *p10* promoters, was introduced in cultured insect cells. The *pH* and *p10* promoter alternated [72]. Recombinant baculoviruses were also used to produce bluetongue virus core (BVC) virus-like particles that expressed the T-cell epitope of the M1 protein of the influenza virus A in order to induce a T-cell response [73]. When the GAG gene was expressed in insect cells, GAG, which is the major nucleocapsid component of retroviruses, displayed authentic antigenic properties and formed virus-like particles that budded from the cell surface into the medium [74]. The L1 and L2 proteins of the human papilloma virus (HPV) capsid produced a capsid-like complex [75]. Insect cells were infected with recombinant baculoviruses producing the herpes simplex virus type 1 (HSV-1) capsid proteins. A virus capsid was assembled when the extracts of infected cells were combined in vitro. Moreover, the purified HSV-1 capsid proteins were capable of procapsid assembly in vitro in the absence of additional factors originating from insect cells [76, 77].

The major structural glycoprotein VP1 of the human polyomavirus (JC virus), which causes multifocal leukoencephalopahy, was synthesized from recombinant baculoviruses in insect cells and proved to form virus-like particles whose morphology was typical of free polyomavirus particles. Purified virus-like particles are highly immunogenic when injected together with an adjuvant and may be used to develop a vaccine [78–80]. Assembly of polyomavirus-like particles was observed in another study using a combination of recombinant baculoviruses to synthesize the virus proteins VP1, VP2, and VP3 [81]. Synthesis of VP2 or VP3 alone does not result in the formation of virus-like particles, but these two proteins are involved in VP1 production and are packaged in virus-like particles.

Kasal et al. [82] expressed the parvovirus major capsid component VP2 in insect cells infected with a recombinant baculovirus, obtained virus-like particles, and used them to vaccinate mammals in order to induce an immune response. Parvovirus-like particles were highly immunogenic in the absence of an adjuvant [82]. Recombinant baculoviruses were used to obtain rotavirus-like particles. Vaccination with purified rotavirus-like particles induced immunity against a heterotypal rotavirus in mammals [83]. Virus-like particles were obtained with the help of recombinant baculoviruses for the hepatitis B and C viruses. Purified virus-like particles were utilized in immunological and therapeutic studies [84–87]. Papillomavirus-like particles were used to develop candidate proteins for constructing a vaccine [71]. Coronavirus-like particles were used to study the assembly of virus particles [90]. My colleagues and I [87] synthesized self-assembled, nonreplicating, genome-lacking virus-like particles of the hepatitis C virus and used them as a virion model to study posttranslational glycosylation of virus coat proteins. Production of influenza virus A hemagglutinin and neuraminidase in insect cells infected with recombinant baculoviruses provides an alternative for the generation of anti-flu vaccines with chicken embryos. Recently, recombinant baculovirus vectors came to be used to obtain retrovirus- and lentivirus-like particles in order to develop a vaccine against HIV [89, 102].

Thus, the set of various recombinant proteins expressed in insect cells grew substantially larger. The set includes cytosolic, nuclear, mitochondrial, membrane-associated, and secreted proteins. The baculovirus expression system is of special utility when it is necessary to obtain functional multisubunit protein structures, such as virus-like particles. The system finds increasing application in developing candidate proteins for vaccines based on virus-like particles and conventional recombinant antigens. Studies with simultaneous expression of enzymes involved in glycosylation, cleavage, and secretion showed that the yield of functional recombinant proteins produced in insect cells can be substantially increased. The use of recombinant baculoviruses to identify the foreign proteins and epitopes exposed on the virus surface provides an efficient means to study the protein–protein

interactions. Recombinant baculoviruses are used to obtain the proteins that occur in minor amounts or the proteins that are difficult to isolate. Such proteins include oncoproteins and proteins contained in the extracellular matrix or involved in intracellular signal transduction. The advantages of the baculovirus system for gene expression in insect cells and its promising improvements created prerequisites for its application for delivering and expressing heterologous genes in mammalian cells in vitro and in vivo.

Baculoviruses As a Means to Deliver and to Express Heterologous Genes in Mammals

The baculovirus expression system attracts particular interest since recombinant baculoviruses have proven suitable for delivering genetic information into mammalian cells [93–99]. Baculovirus genes are transcribed in insect cells, while only genes controlled by mammalian promoters are transcribed in mammalian cells. In insect cells, virus DNA is replicated and packaged into nucleocapsids. In mammalian cells, replication does not take place nor is virus progeny generated.

Hofmann et al. [91] were the first to demonstrate that the AcMNPV baculovirus is capable of transferring genes into cultured mammalian cells, primarily, hepatocytes. Boyce and Bucher [92] confirmed this capability, which still attracts the attention of researchers. Since baculoviruses transfer genetic information into mammalian cells without initiating the production of infectious viruses, recombinant baculovirus vectors are used to develop systems for delivering and expressing functional protein-coding genes in higher animal and human cells in vitro and in vivo [96–98]. The main condition for a baculovirus-delivered gene to be expressed in mammalian cells is that its transcription be controlled by a promoter and regulatory elements that function in these cells. Such elements include internal ribosome entry sites, replication origins, matrix attachment regions, etc. Various regulatory elements active in mammalian cells and inserted in baculoviruses may play a role in regulation to improve the efficiency of gene expression. Transduction of primary hepatocytes and hepatoma cells is efficient and results in high activity of the reporter genes [100, 101]. A new expression system, BacMam, was developed for protein production in mammalian cells. The system utilizes a modified AcMNPV, which is known as a BacMam virus. The virus contains an expression cassette with a promoter functional in mammalian cells and ensures the delivery and expression of the cloned genes in cultured mammalian cells [91, 95, 96]. The BacMam system exerts no toxic effect on mammalian cells, is simple to use, and obviates the need to purify DNA and recombinant viruses for transduction. Recombinant baculovirus vectors most commonly contain expression cassettes under the control of the cytomegalovirus, Rous sarcoma virus, CAG, or SV40 promoter [92, 94, 95, 103]. Hybrid baculovirus vectors were constructed to contain expression cassettes under the control of a mammalian promoter flanked by the inverted terminal repeats (ITRs) of the adeno-associated virus (AAV). Site-specific integration of the AAV genome into the animal cell genome was observed in all cases [104]. Recombinant baculoviruses find increasing application in targeted delivery of genetic information into animal and human specific target cells [105, 106]. For instance, to study replication of the hepatitis C virus, its full-size cDNA controlled by the cytomegalovirus (CMV) promoter was introduced using a baculovirus in Huh7 cells [100]. Assembly of hepatitis C virus-like particles was studied using baculovirus transduction of Hek293T and Huh7 cells with a modified baculovirus carrying cDNAs of the hepatitis C virus structural genes under the control of the CMV promoter [107]. Baculovirus vectors carrying hepatitis B and hepatitis C virus cDNAs are used to study virus infections and to search for new antiviral strategies. Lack of replication and cytotoxicity of baculovirus vectors in mammalian cells makes it possible to obtain high poliovirus titers in a baculovirus system with hybrid T7 RNA polymerase [108].

Mammalian Cell Lines Susceptible to Baculovirus Transduction

Baculoviruse transduction is usually efficient with various cells [95, 96, 98], the highest expression of recombinant genes being observed in cells of a hepatic origin [91]. Studies with a large panel of cell lines, including primary human cell cultures, showed that expression of an eukaryotic gene from a baculovirus is regulated by the CMV promoter and that recombinant baculoviruses are suitable not only for transient expression in mammalian cells but also for generation of stable cell lines, which preserve the expression of the reporter protein after numerous passages. At least 12 kb of the virus genome introduced in Chinese hamster ovarian (CHO) cells persisted in the resulting cell line, although it was unclear whether baculovirus DNA was integrated into the host genome. Transduction of CHO-K1 cells with a recombinant baculovirus containing an expression cassette with the SV40 promoter and the neomycin phosphotransferase gene in the presence of the antibiotic G418 yielded a stable cell line, which expressed GFP for more than 5 months [109]. Another baculovirus vector, which contained two expression cassettes (one with the β-*gal* and hygromycin resistance genes and the other with *rep* flanked by the AAV inverted terminal repeats (ITRs)), efficiently and stably delivered the recombinant genes into CHO cells. The yield of hygromycin-resistant cells was lower in the absence of *rep* and increased 10- to 50-fold in its presence, the AAV genome being integrated into the host genome and the cell survival reaching more than 40% [104].

A recombinant baculovirus with the promoter of the *Drosophila* heat shock protein 70 (*hsp70*) gene and the GFP gene efficiently delivered genes into *Drosophila* S2 cells [63]. Experiments showed that baculoviruses are capable of infecting nondividing cells. The process includes a transport of the baculovirus nucleocapsid through nuclear pores, in contrast to the adenovirus and herpes virus [110]. The possibility to introduce genes into both dividing and nondividing cells in vitro and in vivo is another important advantage of baculoviruses as expression vectors. For instance, a baculovirus was used as a transport vector for stem cells [105]. The set of mammalian cell lines susceptible to baculovirus transduction is continuously expanding, and the transduction efficiency varies from 10 to 90% depending on the cell type.

Efficiency of Baculovirus Transduction

The efficiency of gene expression in less sensitive mammalian cells is not determined by a block of virus penetration into the cell but rather depends on whether the virus is capable of entering the cell nucleus without appearing in intracellular vesicles. A mechanism was proposed to explain the difference in transduction efficiency among different cells [111]. Baculovirus vectors with an increased copy number of sequences coding for gp64 or another coat protein display higher transduction efficiency in mammalian cells [112, 113]. In addition, cell treatment with sodium butyrate or trichostatin A immediately after infection with a recombinant baculovirus increases the expression level by a factor of 20 [95, 114]. Electrostatic interactions between viruses and mammalian cells via cell surface heparin sulfate also affect the efficiency of baculovirus-mediated gene delivery [115]. The efficiency of the delivery and expression of reporter genes in mammalian cells is affected by several other factors, such as phospholipids present on the surface of cells used for transduction [116], a combination of a hybrid baculovirus with another virus (BV + AAV) [104, 117], introduction of the recombinase gene into vector constructs [118], and double transformant selection [119]. The two main AcMNPV transactivators, IE1 and IE2, may activate the baculovirus genome in mammalian cells [129].

Mechanism of the Baculovirus Interaction with Mammalian Cells

Yet little is known on how baculovirus vectors enter mammalian cell lines, as well as on the specificity of virus–cell interactions. The cell surface molecule that interacts with a baculovirus to allow its penetration into the cell is unknown. The saturation kinetics obtained for virus absorption by Huh7 cells during their transduction indicates that the virus binds with a specific receptor on the cell surface. The fact that baculoviruses enter nondividing hepatocytes also suggests specific virus–cell interactions. In contrast, saturation is not observed for baculovirus absorption by HEK293 cells, indicating that the interaction of baculoviruses with these cells is nonspecific. Cell membrane phospholipids are important for the initiation of baculovirus penetration into the cell in the case of HepG2 cells. In addition, virus attachment to the cell surface requires electrostatic interactions, which may be due to heparin sulfate residues exposed on the cell surface. Phosphatidic acid of phospholipids and phosphatidylinositol inhibit transduction of mammalian cells, while heparin and heparin sulfate do not exert such an effect. A possible cause of these discrepancies is that baculoviruses may utilize different mechanisms to interact with different cells. For instance, lysosometropic factors, such as choloquine, inhibit baculovirus gene expression; it seems that acidified endosomes have a means to regulate the processing of virus virions in mammalian cells [91, 95, 110]. It is thought that the AcMNPV gp64 coat protein is necessary for baculovirus–cell recognition and baculovirus entry into the cell. Baculovirus vectors with an extra gp64 gene display higher transduction efficiency in mammalian cells [113].

Delivery and Expression of Heterologous Genes in Mammalian Cells in Vivo

Since baculoviruses are incapable of reproduction in mammalian cells in vitro and humans lack antibody- and cell-mediated innate immune memory against baculoviruses, the baculovirus expression system may find expanded application and is appealing for use in vivo. The system is promising for developing diagnostic and vaccination procedures in gene therapy. The use of recombinant baculoviruses in vivo has certain limitations. The main one is that baculoviruses are neutralized by the complement system upon activation of the C pathway. It was observed that baculovirus vectors administered to rats and mice via various routs, including direct injection into the liver parenchyma, fail to deliver the reporter genes and that baculoviruses are neutralized by a fresh serum but not by a heat-inactivated serum [120]. Experiments with a serum deficient in specific factors of the complement system showed that thermolabile serum factors are responsible for the failure of gene delivery by baculoviruses.

Studies with depleted sera implicated the classical complement cascade in the neutralization of baculoviruses [121–123]. The C barrier to baculovirus-mediated delivery in vivo is overcome by inactivating the complement system or avoiding the contact with the C system. Injection of a recombinant baculovirus in the tail vein of complement-deficient mice allows transgene expression in the spleen, liver, and kidney. A virus was constructed that is resistant to complement in the presence of DAF, which stimulates degradation of complement components [124, 125]. DAF was earlier

demonstrated to increase the degradation rate of the complement system [126]. The virus, which contains the gp64-DAF fusion protein, delivers the human factor IX gene in newborn rats after an intrahepatic injection [113]. Modified baculoviruses that carry a vesicular stomatitis G (VSVG) protein are also complement resistant [127].

Baculoviruses may be employed in transferring genes to sites where their contact with the complement system is avoided. For instance, a direct injection of a recombinant baculovirus into mouse brain tissue results in efficient gene transfer, mostly, in glial cells [114]. To deliver genes in vivo into the rabbit carotid artery, a virus-containing "collar" was placed outside the artery to prevent contact of the recombinant baculovirus with complement [128]. Contact of baculoviruses with the complement system was avoided when a human liver segment was perfused ex vivo. The procedure allowed transduction of baculovirus vectors into liver tissue, demonstrating the possibility of using baculoviruses to transfer genes into mammalian tissues [122].

Thus, the complement system may be blocked using various methods, such as suppression of the complement cascade (with a serum devoid of various complement factors), neutralization (depletion) of C3, serum treatment with anti-C5 antibodies or the cobra venom factor (CVF), the use of the sCR1 regulator (a soluble receptor) of complement activity, the use of the VCP virus component regulator (which inhibits both the classical and alternative pathways of complement activation), virus pseudotyping at VSV-G, and construction of complement-resistant baculovirus vectors. Suppression of the complement cascade protects baculoviruses from serum-dependent inactivation, thus allowing its use as a vector in gene therapy in vivo.

It is necessary to further study the mechanisms that substantially improve the expression of genes put under the control of promoters active in mammalian cells and the factors that affect the reporter gene expression in mammalian cells (such as sodium butyrate or trichostatin A, as well as modification of the baculovirus coat protein gp64 as a means to expand the range of susceptible cells and tissues). Such studies will expand the potential of the baculovirus expression system and its applications.

CONCLUSIONS

The biology of the nuclear polyhedrosis baculovirus, its unique two-phase infectious cycle, and the specific organization of the baculovirus genome provide a potent tool for expressing recombinant proteins in insect and mammalian cells. The principles of transfer vector construction and the advantages of the baculovirus expression system made it possible to obtain eukaryotic expression system that are suitable for producing eukaryotic proteins both in vitro and in vivo.

The baculovirus expression system with insect cells provides a means to study the assembly of virus particles in the absence of an infectious virus and is often used to obtain candidate vaccines based on virus-like particles and conventional recombinant antigens. The set of recombinant proteins produced in baculovirus-infected insect cells is broad and includes various proteins from cytosolic enzymes to membrane-associated proteins.

In view of the advantages of the baculovirus expression system with mammalian cells and the fact that humans lack antibody- and cell-mediated innate immunity against baculoviruses, baculovirus vectors provide a promising alternative to vectors based on human viruses.

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