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The Use of Baculoviruses as Expression Vectors

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ABSTRACT

The use of recombinant baculoviruses as high level expression systems is becoming more and more popular. This review aims to provide a summary of the impact of this expression system in biochemistry and biotechnology, highlighting important advances that have been made utilizing the system. The potential of newly developed multiple baculovirus expression systems to enable the reconstruction of complex biological molecules and processes is also reviewed.

Index Entries: Baculoviruses; signal transduction; multiprotein complexes; insect control; glycosylation.

INTRODUCTION

The last three years have seen a dramatic increase in the use of the baculovirus expression system across a broad spectrum of biological disciplines, including biochemistry, entomology, animal virology, and human physiology (*see* Fig. 1). In addition to these applications, the depth to which the system has been useful within subjects is remarkable, ranging from investigation of single amino acid changes on protein structure to the behavior of large multiprotein complexes.

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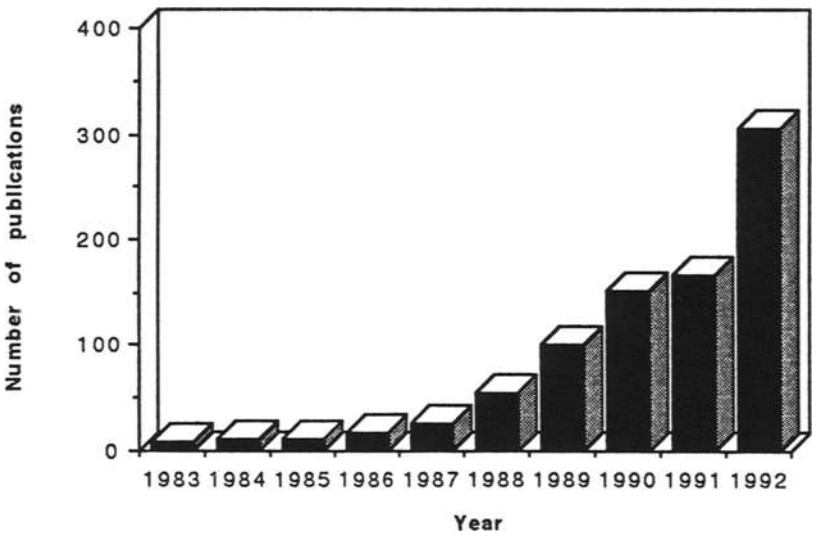

Fig. 1. Histogram of the number of publications containing the keyword "baculovirus" with year of publication. It should be noted that the first reported use of a recombinant baculovirus expression system was in 1983 (*1*). Source of material: Medline CD-ROM.

There are several reasons why the baculovirus expression system has become so popular. Many questions in biology remain unanswered because certain protein molecules are naturally expressed at very low levels. If the genes coding for these proteins are liberated from the restrictions of their natural promoters and placed under the control of a strong promoter, such as those of the polyhedrin or p10 genes of baculoviruses, expression may be raised substantially and the protein used for structural, functional, or diagnostic studies. A further advantage of baculovirus expression, and probably as important as the amount of protein produced, is that insect cells perform almost all the posttranslational processing events found in mammalian systems—in contrast to the situation in prokaryotic systems. Therefore, in virtually all cases, this results in the structural integrity of the folded molecules and full biological function. As well as these advantages over other methods, the baculovirus expression system shares many useful characteristics, such as the speed and ease of production of recombinant viruses—a facet made possible through the relatively recent introduction of modified forms of baculovirus DNA for successful transfection (*vide infra*).

This article will consider the recent developments in the exploitation of baculoviruses for structural and functional protein studies, including their impact on our understanding of intracellular signaling mechanisms. In addition, we shall illustrate their utilization in the production of multiprotein complexes *in vitro*, in vaccine production, and in the provision of

material for diagnostic reagents. The last section is a precis of the continued development of baculoviruses as insect pest control agents.

Exploitation of Baculoviruses

The family Baculoviridae contains several viruses infecting a large range of insect hosts. The prototype virus of the subgroup of multiple nuclear polyhedrosis viruses (MNPV) is *Autographa californica* MNPV (AcMNPV), which naturally infects insects of the same Linnaean name (the fall army-worm) and shows the main characteristic of the group: the production of large intranuclear inclusions in infected cells. Other members of the group include *Bombyx mori* and *Orgyia pseudosugata* MNPVs, which have also been exploited as expression vectors.

For all these viruses, replication occurs in the nucleus of infected cells. Progeny virions are transported into the cytoplasm, where they bud from the cell and are then free to infect other cells. In addition, transmission between insects is enhanced by the polyhedrin protein. Although still in the nucleus, some progeny virions are surrounded by polyhedrin, which aggregates and encases the particles, forming the characteristic intranuclear inclusion bodies. After the death and decomposition of the insect, the polyhedra are deposited on the surface of vegetation, to be ingested by other feeding insects. In this new host, the polyhedra are transported with the food to the midgut of the insect, where the polyhedrin dissolves in the alkaline conditions and the virion particles are released into the lumen. The cells of the midgut become infected to reinitiate the sequence of viral replication.

From the point of view of their exploitation as an expression vector, it is important to realize that baculoviruses have two means of dissemination within the insect population: extracellular nonoccluded virus and occluded virus. Propagation of the virus in insect cell culture renders the polyhedrin dispensible, and hence, its replacement by a foreign gene results in a recombinant infectious virus that can replicate and express the protein product of that gene. Under the control of the polyhedrin promoter, the yield of protein produced should theoretically approach that of the polyhedrin itself, some 30% of total cell protein.

Production of a Recombinant AcMNPV

Methodological reviews are available for the production of recombinant baculoviruses (*2,3*). Briefly, following insertion of the foreign gene into a suitable transfer vector, cotransfection of insect cells with the latter and AcMNPV DNA is achieved by calcium chloride, electrophoration, or lipofection. Recombination occurs between the transfer vector and the AcMNPV genome, in which the gene of interest is inserted into the baculovirus genome in a directed manner. Recombinants may be selected by a number of methods depending on the transfer vector used and the

baculovirus DNA used for transfection (*vide infra*). Once purified, the recombinant is propagated to high titer in insect cell culture.

Although this broad summary is helpful in understanding the underlying concept of the system, it belies the extensive investigations that have been carried out into the natural life cycle and molecular biology of AcMNPV. Much of the pioneering work originated in the laboratory of Summers at the University of Texas A&M, USA (*3,4*), and has been extended by Possee and Bishop at the Institute of Virology and Environmental Microbiology, Oxford (*5,6*). This work has led in turn to a great deal of modification and refinement of the procedures, and the establishment of a range of vectors for the transfer of foreign genes into the wild-type genome. In addition, modifications of the transfecting DNA have increased the efficiency of recombination: for example, the use of linearized AcMNPV DNA in the transfection (*7*) to yield in excess of 30% of progeny virions as recombinants, or in the inclusion of *lacZ* in the genome and the use of conditionally lethal genomes for transfection to yield 100% recombinants (*8,9*). Similarly, the host range of AcMNPV has been expanded by recombination with BmMNPV to allow its replication of both Sf21 cells and silkworm larvae (*10*). Vectors are also available that facilitate the introduction of site-specific mutations. Every opportunity to increase the yield of protein is being taken; for example, the addition of the insect moult hormone 20-hydroxyecdysone to the insect cell culture medium produces a threefold increase in the expression of secreted proteins (*11*).

Construction of Transfer Vectors

Of central importance to the development of the baculovirus expression system is the structure of the transfer vector. Alterations in its composition have led to considerable improvements in the quality and amount of protein produced. There is now a plethora of transfer vectors available for different purposes from both academic and commercial fields (reviewed in [*6*]). The majority of high-level expression vectors contain the entire polyhedrin leader sequence intact, up to and including the "A" of the polyhedrin ATG translation start codon (e.g., pAcYM1 [*5*]). Many transfer vectors have been synthesized to facilitate more rapid screening and selection of recombinants. Recognition of recombinant plaques for selection was the rate-limiting step, and hence, expression vectors containing the *lacZ* gene have been produced (*12,13*). Thus, scanning transfection mixtures for recombinant virus is made considerably easier. However, in these cases, the reporter enzyme continues to be expressed after its primary function is complete (i.e., after selection of the recombinant).

Alternative recombination sites in the AcMNPV genome have been used, for example, the p10 gene. This is useful since the polyhedrin gene is essential for the efficient infection of insect larvae. The p10 gene appears to be nonessential for viral replication, and protein expression from this

site is equivalent to that from the polyhedrin site (*14*). Multiple expression vectors based on duplication of the important polyhedrin transcription areas, and combinations of p10, polyhedrin, and basic protein promoters have also been constructed (reviewed in [*6*]). These multiple expression vectors can be used to produce two different proteins in insect cells, and overcome the problems associated with the low efficiency of coinfecting cells with two or more recombinant viruses. More recently, a range of AcMNPV multiple transfer vectors has been described that offers differential expression of two genes by employing a different transcription promoter for each gene (*15*). In addition, the same report describes a series of related plasmids that allow insertion of a foreign gene in any of three translational reading frames and, on ligation of the gene of interest, supply an N-terminus with an efficient translation initiation signal. This supersedes the use of fusion vectors necessary for genes in which an initiation codon is absent, although these fusion vectors are still useful for cases where products of translation are unstable (*16,17*).

Other limitations of the baculovirus system are gradually being overcome. In general, foreign proteins that are normally secreted are produced in lower quantities than membrane-bound molecules, which in turn are produced in lower amounts than structural proteins. However, this bias toward structural proteins may be related to the polyhedrin locus itself. Recent work has shown that incorporation of the signal sequence of honeybee melittin (a secreted insect protein) into the cloning site of the transfer vector (pVT-Bac) resulted in a fivefold increase in the secretion of a heterologous reporter enzyme (*18*).

Posttranslational Processing of Proteins

Despite authentic site-specific glycosylation within the insect cell, a frequently observed characteristic of the baculovirus expression system is the inability of insect cells to produce the complex processed carbohydrate patterns seen on some mammalian and viral glycoproteins, for example, HIV gp120 and influenza virus hemagglutinin (*19–21*). Thus, the oligosaccharides remain in a partially processed high-mannose form. However, Davidson and colleagues have published a series of investigations showing that insect cells have the capacity to process carbohydrate on some proteins. Initial investigations (*22*) of baculovirus-produced human plasminogen demonstrated that 40% of the carbohydrate was present as complex oligosaccharide. This proportion was structurally identical to the human protein, indicating that trimming and processing of the high-mannose sugar chains into complex oligosaccharide could occur in insect cells. These data suggest that either amino acid configurations or protein conformational properties can influence oligosaccharide processing. Subsequent studies (*23–25*) showed that the nature of N-linked glycosylation was dependent on the length of time postinfection. At late

times postinfection (60–96 h), 92% of the total oligosaccharides were of the biantennary, triantennary, and tetra-antennary complex classes with varying extent of outer-arm completion (*25*). These data clearly indicate that insect cells possess the glucosyltransferase genes necessary for complex oligosaccharide processing and offer the prospect of manipulation of the baculovirus expression system to mimic the full glycosylation pathways observed in mammalian systems. It is worth noting that despite aberrant glycosylation, the majority of proteins expressed in the baculovirus system show identical biological activity to their fully processed counterparts.

Other studies have investigated the authenticity of other posttranslational modifications, such as phosphorylation (*26*). Although insect cells were able to phosphorylate SV40 virus T antigen to a qualitative extent similar to that of native protein, the amount of phosphorylation was different. More specifically, serine residues appeared to be underphosphorylated although without affecting the biological functions of the protein. Studies on *ras* and *ras*-related proteins produced in the baculovirus expression system (*27*) have shown that correct isoprenylation and palmitoylation were performed by the insect cells. Indeed, the membrane association of the proteins was dependent on isoprenylation. Similar work on the expression of influenza virus hemagglutinin by recombinant baculoviruses (*28*) showed that palmitoylation correctly occurred in the posttranslational processing.

PRODUCTION OF PROTEINS FOR STRUCTURAL STUDIES

The baculovirus system has proven particularly useful in the generation of large quantities of protein for structural analysis (*29–34*). In many cases, the naturally produced proteins encoded by predicted open reading frames (ORFs) have not been detected because they exist in such low concentrations. In addition, those that have been cloned and expressed in bacterial expression systems often lack conformational integrity.

As an illustration (*30*), an ORF present on the human chromosome 17 coding for a polypeptide to a new class of lymphocyte activation protein has been cloned and expressed in the baculovirus system to yield a 10-kDa recombinant protein. Monoclonal antibodies against the expressed protein were produced and subsequently used to precipitate a 14-kDa protein from extracts of activated natural killer cells. Despite the differences in molecular size, which probably reflect incomplete glycosylation by the insect cells, the recombinant and precipitated proteins were identical and should enable further investigations into the functional characteristics of this novel protein to occur.

Another notable example (*33*) concerns the rotavirus genome segment 11, where the nucleotide sequence shows three alternative reading frames, one of which is the primary protein product NS26. However, one of the remaining ORFs contains a strong initiation codon, implying that the protein from this reading frame could also be produced in infection. Using a recombinant baculovirus, this out-of-phase ORF was expressed in insect cells, and specific antibodies raised against the expressed protein product allowed the presence of the protein to be formally demonstrated in rotavirus-infected cells.

The ability of the baculovirus system to generate large amounts of protein has also led to its utility in the determination of the three-dimensional structure of proteins. Despite the high-mannose glycosylation of recombinant polypeptides in insect cells, the conformation of the folded molecules is invariably authentic. Therefore, methods of structural analysis requiring large amounts of protein, such as electron microscopy or X-ray diffraction, can readily be performed. For example, adenovirus penton fibers have been expressed in the baculovirus system, and by electron microscopy were shown to be morphologically identical to those extracted from cultures of wild-type virus (*35*). Similarly, genes coding for human hepatitis B core (*36*), rotavirus (*37*), HIV-1 (*38,39*), and bluetongue virus (*40–43*) capsid proteins have been expressed, and the recombinant proteins observed to self-assemble into empty capsids with correct conformation (*vide infra*).

A notable example of X-ray structural analysis of baculovirus expressed protein is the determination of the structure of human parvovirus B19, a virion particle with predicted icosahedral symmetry. The major capsid protein VP-2 produced by a recombinant baculovirus self-assembles into empty capsids, which have been crystallized and subjected to X-ray diffraction. Preliminary data at 9 Å resolution indicate a space group of P2(1)3 with a = 362 Å (*44*). The more refined three-dimensional structure is awaited with interest. However, other similar studies have not been so successful. In an investigation of the ability of the adenovirus E3/19K protein to block cell-surface expression of human HLA class I molecules, assembly of the class I heavy-chain molecule with β-2-microglobulin to form the fully functional heterodimer was inefficient (*45*). Although the reasons for these results were unclear, incomplete glycosylation of the HLA class I molecule was suggested. Nevertheless, other studies using murine HLA class I heavy-chain molecules have shown that assembly of the former with murine β-2-microglobulin could be achieved to a significantly higher degree, and that the HLA class I heterodimer was structurally authentic as determined by the binding of conformation-dependent antibodies (*46*). Furthermore, the efficient assembly of the heterodimer was dependent on the presence of the appropriate HLA-restricted peptide, a further explanation for the poor results observed in

the aforementioned study (*45*), which did not include the appropriate HLA-restricted peptide to direct assembly.

PRODUCTION OF PROTEIN FOR FUNCTIONAL STUDIES

In recent years, the baculovirus system has been extensively employed for the expression of a diverse range of proteins that have been shown to be functionally authentic. These range from the production of biologically active hirudin, a secreted anticoagulant from the leech (*47*), to the investigation of various enzyme functions and specificities, e.g., papain (*48*), cytochrome P450 (*49*), human acid-β-galactosidase (*50,51*), and aldose reductase (*52,53*).

The potential offered by the baculovirus expression system for future improvements in the treatment of human disease is aptly demonstrated by the following studies. Human acid-β-galactosidase (HABG) has been shown to be defective in Gaucher's disease, but the condition has a complex genetic background, with multiple amino acid substitutions being responsible for enzyme dysfunction. Using site-directed mutagenesis, base changes coding for amino acid substitutions Arg with Gln at amino acid position 120 and Asn with Ser at position 370 duplicate the enzymatic dysfunction (*50*). This region encompasses residues critical for catalytic active site formation, suggesting a physical basis for the paucity of function in the Gaucher-type enzyme. A more recent study by the same group has extended these observations to types 2 and 3 Gaucher disease (*51*), showing that similar amino acid substitutions could provide the molecular basis for disease, and that the severity of disease is linked to the activity of the mutated enzyme. Aldose reductase is an enzyme involved in the complications of diabetes, and the control of the activity of this enzyme has potential in the management of the diabetic patient. Two groups have cloned and expressed aldose reductase using the baculovirus expression system (*52,53*), and both sets of data show that the expressed enzyme is functionally identical to the native enzyme. This should prove ideal material for the screening of aldose reductase inhibitors for therapeutic use.

Human β nerve growth factor (NGF) has been cloned and expressed using a recombinant baculovirus, and characterized (*54–56*) in an effort to identify therapeutic agents for Alzheimer's disease. NGF may have potential in reversing neuronal degeneration, which is a feature of Alzheimer's and other conditions. The recombinant nerve growth factor promoted neurite outgrowth in an in vitro assay system using cultured neuronal cells, induced a differentiation response, and protected the cells from degeneration following a deliberately induced lesion. More recent studies have shown that binding to NGF to the cell receptor resulted in a signifi-

cant increase in the dopamine content of the cultured neurons and that NGF exists in three stable dimeric conformations with distinct biological activities (*57*). With the advent of gene therapy, these types of study indicate that such biochemical diseases may not be refractory to treatment.

G Protein Signal Transduction Pathways

Several groups have reported the suitability of the baculovirus expression system for the production of G protein subunits or for the investigation of the effect of G protein activation on intracellular signaling. Individual G protein subunits have been cloned and expressed in the baculovirus expression system (*58*). Recombinant viruses encoding a β and a γ subunit were used to coinfect insect cells, and the dimers were capable of catalyzing the ADP ribosylation of a separately cloned α subunit. Neither the β nor the γ subunit alone was capable of this function. The G protein α subunit is itself composed of several individual subunits. In a separate report from that mentioned above (*59*), the same workers expressed, purified, and characterized the Gi1, Gi2, and Gi3 subunits; these were expressed at approx 2 mg/300 mL of cell culture fluid. The purified subunits were shown to behave in a comparable fashion to their natural counterparts, interacting with angiotensin receptors and restoring the high-affinity ligand binding state in receptors experimentally modulated to a low-affinity state.

Further down the signal transduction pathway, adenylyl cyclase has been cloned and expressed in the baculovirus expression system, and exhibited similar structural and behavioral characteristics to that purified from bovine brain tissue (*60*). The adenylyl cyclase molecule was inserted into the insect cell membrane and could be activated by the G protein α subunit, calmodulin and adenosine analogs, and inhibited by the G protein β/γ subunit. Within the phosphatidylinositol phosphate pathway, the mouse calcium/calmodulin-dependent protein kinase α subunit has been expressed, at a level of 12–15 mg/L of insect cell culture fluid (*61*). After purification, structural and functional analysis showed the product was identical to that purified from rat brain. Interestingly, the recombinant enzyme subunit was able to generate calcium-independent autophosphorylation; a functional feature of the native enzyme.

A particularly significant study that coalesces the major aspects of signal transduction is the cloning and expression of the β-adrenergic receptor of the turkey (TBAR; [*62*]). The receptor was purified from the insect cell culture supernatant and inserted in phospholiposomes together with various G proteins. Following binding of known TBAR agonists, the recombinant receptor catalyzed the activation of the Gs subunits to GTP to the same degree as natural TBAR. In addition, the recombinant receptor was able to stimulate adenylyl cyclase activity in response to the same agonists.

Tyrosine Kinase-Associated Mechanism

The tyrosine kinase route of intracellular signal transduction is relatively poorly understood. Nevertheless, the baculovirus expression system is proving to be a valuable tool in the elucidation of the steps involved. Recent work on the human epidermal growth factor receptor demonstrates its utility. The intracellular domain (EGFR-IC) possesses the kinase activity that may affect the behavior of a responsive element mediating transcription. It has been suggested that following binding of epidermal growth factor to the extracellular region, the receptor undergoes a conformational change resulting in autophosphorylation of tyrosine residues in the intracellular domain, thus activating the kinase action. Several groups have reported the cloning and expression of the EGFR-IC domain (*63,64*), with purification to 95% homogeneity using a simple three-step chromatographic method. Yields of 3–4 mg of pure EGFR-IC could be achieved from roller culture and 20 mg produced using more sophisticated bioreactor techniques (*64*). Using the recombinant material, the former study (*63*) showed that the protein was autophosphorylated at the same sites as wild-type EGFR, and that the autophosphorylation mechanism was capable of phosphorylating synthetic peptides containing residues identical to the main autophosphorylation site of the native enzyme. This occurred more readily in the presence of Mn^{2+} than Mg^{2+}, and kinase activity was retained throughout the purification procedure. The same research group has extended these studies to probe the specific interactions of the kinase region and its control by phosphorylation (*34*). The data showed that phospholipase C-gamma associates with EGFR-IC only if the latter has been authentically autophosphorylated, and is dependent on a specific tyrosine residue at amino acid 771. This conclusion was strengthened when synthetic peptides containing the phospholipase C-gamma site and tyrosine 771 were also phosphorylated by the recombinant EGFR-IC. Therefore, not only was the activity of the kinase domain of EGFR-IC increased by autophosphorylation, but EGFR-IC was capable, once in an activated state, of specifically phosphorylating other proteins, such as phospholipase C-gamma. Some aspects of this work have been independently confirmed (*64*), and the success of these types of study offers the prospect of elucidating the complete mechanism of signal transduction using defined expressed products in an in vitro environment.

One other aspect of signal transduction is worthy of mention—that of oncogenes. Many natural molecules have oncogenic counterparts, and the tyrosine kinase receptor is no exception. The rat *neu* oncogene product has close structural homology to the EGFR. The oncogenic effect of the *neu* protein is mediated by a point mutation within the transmembrane region. A recent report (*65*) described the expression of a variety of forms of *neu* protein. Results showed that when the transmembrane portion of *neu* was absent, the kinase activity of the intracellular portion was at its highest, implying that the transmembrane region exerted a sup-

pressive effect on the enzymatic activity of the intracellular region. However, if an amino acid substitution from V to E at position 664 was introduced into the transmembrane region (the mutation observed in the oncogenic molecule), the suppressive effect of the transmembrane region was removed, and the kinase activity regained its former activity through an improved ability to utilize Mg^{2+} rather than Mn^{2+}.

Ion Channel Mechanisms of Signal Transduction

These are the least understood of the signal transduction mechanisms, and hence, fewer studies have been performed. Probably the most significant in medical terms is the discovery that the cystic fibrosis gene product (CFTR) is likely to produce a defective ion channel. One of the characteristics of the disease is an oversecretion of mucus by epithelial cells, and the hypothesis is that this is normally regulated by an ion channel system. The CFTR has been expressed by a recombinant baculovirus (*66*) and induces the insect cells to undergo a change in cyclic-AMP stimulated permeability, a characteristic observed in CFTR-expressing human epithlial cell lines.

Of more academic interest, and clearly in its infancy, is the application of baculovirus expression systems to investigate the physiology of ion channel regulation of neuron conductance. Two reports (*67,68*) have investigated the behavior of Shaker K^+ currents induced in insect cells by infection with a recombinant baculovirus containing the gene coding for the Drosophila ion channel. One study (*68*) examined the kinetics of the appearance of Shaker currents and identified a 75-kDa protein that appeared to be responsible for the electrical conductance properties of the insect cells.

Other Membrane Transport Proteins

These types of protein have been successfully obtained in large amounts using the baculovirus expression system. The multidrug transporter protein MDR1 is responsible for the removal of a number of cytotoxic agents from the cell, including drugs that are often prescribed for treatment of tumors. MDR1 has been cloned, expressed, and characterized, and although not properly glycosylated, is biochemically identical to forms of the protein isolated from multidrug-resistant cell lines (*69*). The recombinant protein was phosphorylated and appears to act as an efflux pump with similar specificity for the expulsion of cytotoxic drugs to the native protein. Such results are promising for the future elucidation of the mechanism of action of these proteins.

Similar observations have been made for other membrane transport proteins: the Na^+/H^+ antiporter molecule, which is responsible for the simultaneous exchange of Na^+ and H^+ across the cell membrane (*70*),

and the D-glucose transporter GLUT-1 (*71*). The baculovirus expression system again proves to be ideal for the biochemical investigations of these molecules, showing in the first report an increase in the Na^+ influx in response to an external acidification stimulus (*70*) and, in the second report, a large increase in the number of D-glucose-sensitive binding sites on the insect cell surface (*71*). In both cases, these observations parallel the synthesis of recombinant protein. In the case of the Na^+/H^+ antiporter molecule, although only 10% of the protein was estimated to be fully functional, the behavior of this proportion was identical to the predicted phosphorylation, signaling, and transport characteristics. Taken together, these examples open the way for the molecular dissection of this class of transporters, especially with the opportunity to produce truncated forms and site-directed mutants to elucidate critical binding and catalytic sites.

Production of Monoclonal Antibodies

Perhaps the best testimony to the usefulness of the baculovirus expression system is the production of fully functional antibody molecules from cloned immunoglobulin genes. Two studies are noteworthy. In the first (*72*), murine immunoglobulin heavy and light chains were coexpressed by dual infection of insect cells with recombinant baculoviruses separately coding for the two polypeptide chains. Structural and functional analysis indicated that the two chains correctly assembled to form the heterodimer, and that both monoclonal and polyclonal idiotypes were present. The second study (*73*) reported similar findings, but also demonstrated that the expressed antibody molecule-mediated specific reactions with other components of the immune system, e.g., antibody-dependent cell-mediated cytotoxicity and specificity of interaction with heterologous sources of complement. These types of investigations highlight the advantages of the baculovirus expression system to produce functional monoclonal antibodies and allow the relatively facile mutagenesis of such antibodies. The generation of mutant antibodies by hybridoma technology is difficult, and the hybridoma cells cannot be relied on to reproduce antibodies with these specific mutations *ad infinitum* because of recombination events within the hybridoma cell. In addition, hybridoma cultures eventually lose their ability to produce antibody as a result of the overgrowth by faster expanding clones of nonsecreting cells.

ANTIGENIC STUDIES

The baculovirus expression system has proved to be a valuable tool in the synthesis of recombinant proteins for immunization against infection,

modulation of disease, diagnosis of infection, and the dissection of the immune response to infectious agents. There are many microbiological infections for which immunization is desirable but difficult to achieve, especially if the agent cannot be grown in vitro or if propagation is hazardous. These facts are true of many of the zoonotic and arbovirus agents encountered in the tropics, and vaccines against these diseases are becoming more economically desirable as the countries visited by Western travelers become more exotic and political pressure increases the profile of underdeveloped countries.

Several recombinant proteins that have been produced using baculovirus expression systems have excellent potential as vaccines. These include vaccines against Hantaan virus (*74*), dengue virus type 1 (*75*), rabies virus (*76*), bluetongue virus (*77*), parvovirus (*78*), human immunodeficiency virus (HIV) (*79*), anthrax (*80*), malaria (*P. falciparum* (*81*)), and *Clostridium tetani* (*82*). All of these have elicited a strong neutralizing antibody response postvaccination and have had varying degrees of success in protecting the immunized animal. Some infections are more refractile to protection through immunization because they are more complex in pathology, but here too the use of baculovirus expression system has generated encouraging results. For example, in respiratory syncytial virus (RSV), immunization with the major envelope protein of the virus paradoxically results in more severe disease on challenge compared with nonvaccinees, probably as a consequence of antibody enhancement of infection. However, vaccination of animals with a baculovirus-produced chimeric protein consisting of the RSV fusion and attachment proteins showed that little or no immunopathology occurred on subsequent challenge of the animals (*83*).

In order to delay the onset of AIDS in HIV-infected patients, baculovirus-produced CD4 molecules that bind to the HIV-1 gp120 have been used (*84*). In these studies, the CD4 molecules were electroinserted into erythrocyte (RBC) carriers. On subsequent examination, the CD4-RBC had an in vivo half-life of 7 d, and was able to aggregate T-cells chronically infected with HIV-1. Also, in vitro work showed that preincubation of HIV-1 with RBC-CD4 reduced the appearance of HIV antigens and viral reverse transcriptase activity in subsequent cell cultures by 80–90%. These data are encouraging for the future of therapeutic strategies aimed at delaying the onset of AIDS. Vaccines against HIV have also been produced using the baculovirus expression system. Recombinant gp160 elicited a specific antibody response and lymphoproliferative responses in vitro showed the induction of memory T-cells (*85*) without compromising immunocompetence (*86*). The first clinical trial using baculovirus-produced gp160 was announced in 1987 by MicroGenSys, Connecticut (*79*). The results of phase II clinical trials using priming with vaccinia virus-produced gp160 followed by boosting with baculovirus-produced gp160 are awaited.

Dissection of the Immune Response

In many infections, the immune response of the host is not completely understood and remains an obstacle to the production of useful vaccines. Previously, immunization has been carried out using crude preparations of material from large-scale culture, and although some protection has been achieved in a number of cases, this may be at the expense of severe reactions to other components of the vaccine. In all cases, it is crucial to understand the nature of the immune response to natural infection in order to design more effective and safe vaccines. By way of illustration, Roy and colleagues (*77*) have investigated the immune response of sheep to bluetongue virus. Bluetongue viral proteins expressed from recombinant baculoviruses were administered in different quantities and combinations to sheep, whose antibody responses were measured and then challenged with wild-type bluetongue virus. Results indicated that the outer capsid protein VP2 was effective at protecting a proportion of sheep following challenge, if < 50 μg/sheep were given, and all of them if more than 50 μg were used. However, administration of 20 μg/sheep of the other outer capsid protein VP5 at the same time as the 50-μg dose of VP2 resulted in the complete protection of all sheep with a concomitant increase in the neutralizing antibody response. Addition of four viral core proteins or three nonstructural proteins to the vaccine preparation did not enhance the neutralizing antibody response.

Similarly, the immune response of infant rhesus monkeys to rotavirus protein VP4, and its two cleavage products VP5 and VP8 (*87*) have been investigated. Both these polypeptides contain the major epitopes for neutralization found in most (VP5) rotaviruses or are strain-specific (VP8). Thus, an ideal candidate vaccine should elicit a strong immune response to the common neutralization antigens. However, using baculovirus-expressed recombinant proteins in an assay to measure antiVP5 or antiVP8 responses in animals vaccinated with rhesus or rhesus/human rotavirus pseudotypes, it was found that VP5 was poorly immunogenic, indicating that any effective vaccine must be VP8 strain-specific. Other work on rotavirus immunity has focused on the cellular immune response to infection (*88*). Using a severe combined immune defiency (SCID) mouse model, it has been possible to demonstrate that adoptive transfer of T-lymphocytes from immunized histocompatible mice can mediate clearance of a persistent murine rotavirus infection in these animals. This clearance was not dependent on the immunizing rotavirus type, and immunization of donor mice with baculovirus-expressed recombinant proteins showed that only some of these rotavirus proteins were capable of mediating clearance of infection in the recipient mouse.

Diagnostic Reagents

As in the case with vaccine development programs, the preparation of reagents for diagnostic testing has been an area where the baculovirus

expression system has made significant contributions. Examples of diagnostic reagents for dangerous pathogens include Lassa fever virus nucleoprotein (*89*), rabies virus nucleoprotein (*90*) and HIV p24 core protein (*91*). In the Lassa fever virus study, the baculovirus-produced nucleoprotein was indistinguishable by SDS-PAGE and western blotting with a variety of monoclonal antibodies and polyclonal immune sera. Crude recombinant virus-infected insect cell lysates were used to develop a sensitive and specific enzyme immunoassay for Lassa fever virus antibodies, and a fluorescence test was developed and validated using the infected insect cells as a substitute for mammalian cells infected with Lassa fever virus. An infection worthy of note is human parvovirus, which is a recently characterized agent responsible for erythema infectiosum in the general population, and a more severe aplasia in individuals with hemolytic anemia. The virus has proven difficult to grow in tissue culture, and so the only source of antigen for serological tests has been that purified from donated blood. In 1990, Brown and colleagues (*92*) reported the production of VP1 and VP2 of parvovirus B19, and their suitability as a diagnostic reagent in an immunofluorescent assay.

MULTIPROTEIN COMPLEXES

The previous sections of this article have surveyed the structural and functional capacities of recombinant proteins produced in the baculovirus expression system. The majority of these investigations have been conducted on singly expressed products. Because of the increasing availability of multiple expression vectors, studies on protein-protein interactions are now possible, and more recently construction of whole biological systems of interacting proteins has been achieved.

Virus Core Assembly

Expression of the feline and human immunodeficiency virus gag and pol genes using the baculovirus expression system has enabled the development of a biological system in which protease inhibitors may be assessed (*38,39*) and the dissection of virus-like particle assembly (*93*). Since the HIV-specific protease is essential for the production of infectious virions, compounds that inhibit the protease are of interest as antiviral agents. The first study (*38*) developed an intracellular assay system in which the putative protease inhibitor compounds were added to the culture medium of insect cells infected with recombinant baculoviruses expressing the gag and pol genes of HIV-1 and HIV-2. These studies show that protease inhibitors affected the maturation of the gag protein into the core subunits and therefore the assembly of the viral core itself. The other investigation (*39*) used seven recombinant baculoviruses coding for complete or truncated forms of the core polyprotein to delineate the functional domains

required for assembly. Using seletive deletion or addition of karyophilic signal sequences, it was shown that the p6 region was intimately involved in the transport of the core particles to the nucleus.

To date, some of the most elegant baculovirus expression studies concern the self-assembly of viral capsids. Roy and colleagues have made a major contribution in this area (*43*) with their studies on bluetongue virus. They have successfully coinfected insect cells with recombinant baculoviruses coding for no less than seven structural proteins. These assembled to form viral core-like particles on which structural analysis could be carried out, thereby providing a system in which to determine conservation of interacting sites on structural proteins between related viruses. This group has previously demonstrated that two (*40*), four (*41*), and five (*42*) bluetongue virus proteins can be expressed in the baculovirus system and assemble into single- or double-shelled structures. Similar production of morphologically intact structures has been demonstrated with parvovirus B19 (*44,78,94*), bovine rotavirus (*37*), and Newcastle disease virus (*95*).

Regulation of Transcription

All signal transduction pathways, whether G protein, tyrosine kinase, or ion channel-mediated, eventually exert an effect on cellular transcription, and the elucidation of the mechanisms of transcriptional control is of paramount importance. This is especially true with regard to interference with the normal processes by factors that operate directly on transcription, such as oncogene products and viral *trans*-acting factors. Several investigations have employed the baculovirus expression system for these purposes, studying normal cellular transcription (*96,97*) or viral control of transcription (papillomavirus E1 and E2 proteins (*98*), HIV-1 rev protein (*99*), HTLV rex protein [*100*]). For example, the two components of the AP-1 transcription activator protein complex, c-*fos* and c-*jun*, have been cloned and expressed using recombinant baculoviruses in insect cells (*96,97*). After characterization, a system was developed to investigate their transcriptional enhancement in vitro. Binding experiments showed that c-*jun* specifically binds to DNA through an AP-1 specific binding site; c-*fos* alone did not bind to this site. However, when both proteins were present, a significant increase in the affinity of c-*jun* for the binding site was noted. It was also shown that c-*jun* was capable of initiating RNA synthesis if the AP-1 site was present and that addition of c-*fos* stimulated the transcriptional activity of the complex.

Control of DNA Replication

Several groups have used the baculovirus expression system to unravel some of the intricacies of viral replication. In viruses with a relatively high coding capacity, such as herpes simplex virus type 1 (HSV-1), it is known that replication of the viral genome is usually performed by a complex of

several proteins, but until recently it has been difficult to ascribe particular functions to specific proteins within the complex. Two related independent investigations on the DNA polymerase and the DNA primase/helicase complexes of HSV-1 replication illustrate the utility of recombinant baculovirus (*101,102*).

The HSV-1 DNA polymerase consists of two polypeptides, UL30 (the catalytic polymerase) and UL42 protein, which associate specifically to form the fully functional enzyme. One report using baculovirus-produced components (*101*) has shown that the UL30 catalytic subunit was less efficient than the heterodimer at utilizing primers on single-stranded DNA templates. Addition of the UL42 subunit restored the efficiency of the enzyme, providing evidence that UL42 protein acts with UL30 to increase the processivity of polymerization. These findings have been confirmed and extended (*102*) to show that a DNA-binding protein, UL29, is also required to remove local regions of secondary structure that may interfere with the cooperative action of UL42.

The HSV-1 primase/helicase complex consists of the protein products of the UL5, UL8, UL52 genes. Baculovirus expression systems have been used to express all three proteins separately, and to study the effects of omission of particular components on the DNA-dependent ATPase and DNA-unwinding activities of the complex (*102*). Insect cells coinfected with the recombinants' exhibited ATPase activity and unwinding of DNA, implying that the complex had self-assembled within the insect cells and that UL8 was not necessary for full activity. Other data (*103*) have shown that DNA primase and DNA-dependent GTPase activity could also be accomplished by the UL5:UL52 complex without the assistance of the UL8 component. Further work using recombinant baculoviruses has shown that UL8 acts to increase the efficiency of primer utilization by stabilizing the association between newly synthesized oligoribonucleotide primers and template DNA.

The pinnacle of such studies on HSV-1 replication has been reached by Stow (*104*), in which the 7-HSV-1 proteins required for viral origin-dependent DNA synthesis have been expressed using baculovirus and a mixture of the seven recombinants used to produce origin-dependent replication of DNA in the insect cell. Of particular interest was the demonstration of concatameric replicative structures that are also seen in HSV-1-infected mammalian cells and so support the rolling circle mechanism of viral replication seen in the *Herpesviridae*.

INSECT CONTROL STUDIES

Although wild-type baculoviruses have been used as efficacious pest-control agents for many years, they have a limited host range, and can take between 3 and 7 d to debilitate the insect larvae. However, they have

considerable potential, especially since they can be applied to crops by spraying, and so there have been many modifications aimed at increasing this efficiency. The majority of these studies have centered on the replacement of the polyhedrin or p10 genes with heterologous insect toxins. A major effect of these toxins is that they cause the cessation of feeding in the insect much more rapidly after infection than that observed in wild-type virus infections, regardless of the time of death.

Several different toxins have been cloned into recombinant baculoviruses (*105–108*). As discussed in the Introduction, transmission between insects is enhanced by the presence of the polyhedrin protein, and so more recent studies have used the p10 promoter to drive toxin production while maintaining the intact polyhedrin. Toxins incorporated into recombinant baculoviruses include the δ-endotoxin of *Bacillus thuringiensis* (*108*), the insect-specific toxin from the Algerian scorpion, *Androctonus australis* (*105,107*), and the neurotoxin gene of the mite, *Pyemotes tritici* (*106*). In all studies, the toxin resulted in a shorter time to cessation of feeding than observed with wild-type baculovirus-infected insects. It is interesting to note that the scorpion toxin exerts its effect by interfering with Na^+ ion channels in insect neurons, producing rapid paralysis and subsequent death of the insect relatively quickly, and is the mode of action of most chemical-based insecticides. Additionally, it has no effect on mammalian neurons, which makes it an ideal candidate for an improved insect control agent.

CONCLUSIONS AND FUTURE PROSPECTS

In this article, we have attempted to illustrate the utility of baculovirus expression vectors in many areas of biology, and to highlight subjects in which significant advances have been facilitated by the availability of the expressed products. The fact that the system is becoming so widely used is a testimony to its general usefulness and reliability. The rapid expansion of its use is a consequence of the recognition that the production of large quantities of pure, functionally authentic protein has now become a relatively straightforward stage in a research program, rather than a major time-consuming process. Nevertheless, there are still many advances to be made, and it is clear that the system has yet to reach its full potential. The prospect of simultaneous production of 10 or more proteins may offer the biochemist and technologist the opportunity to unravel complex multiprotein processes in a single defined system, or to produce multicomponent vaccines with structural integrity.

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