The Baculovirus-Infected Insect Cell as a Eukaryotic Gene Expression System

M. J. FRASER

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1 Introduction

Baculovirus-infected insect cell cultures are established as an easily manipulated eukaryotic system for highly efficient expression of gene products. The system takes advantage of several unique attributes of this virus group, including highly active late gene promoters, the capacity for insertion of large fragments of foreign DNA, replication competence of the resulting recombinants, and the relative ease of handling both the insect cell cultures and the viruses.

Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556, USA

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This review will attempt to give an overview of the use of baculoviruses as expression vectors, focusing on the *Autographa californica* nuclear polyhedrosis virus as the prototype virus system. The available evidence for proper and improper post-translational processing, cellular localization, and antigenicity of various protein products will be discussed. Several of the currently employed expression vectors are described, and several of the key techniques used in generating baculovirus recombinants will be reviewed. For additional information on baculovirus expression vector constructs, handling the viruses and cell cultures, and analyzing recombinants the reader is referred to previous reviews by MILLER et al. (1986), SUMMERS and SMITH (1987), LUKOW and SUMMERS (1988), KANG (1988), MILLER (1988), CAMERON et al. (1989), VLAK and KEUS (1990) and LUCKOW (1991).

2 The Biology of Baculoviruses

The insect-pathogenic nuclear polyhedrosis viruses (NPVs) are members of subgroup A of the family Baculoviridae. These DNA viruses replicate within the nuclei of susceptible insect cells and have a complex, essentially biphasic replication cycle that generates two infectious forms, extracellular budded virus (ECV), and occlusion bodies (OBs). The two infectious forms of the virus are genotypically identical but phenotypically distinct, each serving a vital function in the survival of the virus in host insect populations. The OBs are an environmentally stabilized form of the virus that function to initiate the primary infection within the gut of host insects, while the ECVs serve to disseminate the virus between cells in the insect host and are employed during all manipulations of the virus in vitro.

The NPVs may be further subdivided based upon the arrangement of nucleocapsids in the occluded virions within OBs. The M-type viruses occlude enveloped single and multiple nucleocapsids, while the S-type viruses occlude only enveloped single nucleocapsids. The prototype virus for the M-subtype is AcMNPV (MATTHEWS 1982), while the S-subtype is represented by the *Heliothis zea* NPV (HzSNPV; CORSARO and FRASER 1987a).

The following discussions are meant to serve as a general introduction for those unfamiliar with baculovirus replication. For more detailed discussion of baculovirus biology and genetics the reader may consult recent reviews (DOERFLER and BOHM 1986; GRANADOS and FEDERICI 1986; BLISSARD and ROHRMANN 1990).

2.1 Viral Life Cycle

During the first phase of the lytic infection (0–20 h) the rod-shaped nucleocapsids are assembled within the nucleus of infected cells in pockets of the virogenic

matrix (FRASER 1986a). Electron-microscopic observations suggest that the bulk of the rod-shaped capsid sheath assembles prior to incorporation of the circular 128-kb virus genome, and that extension of the capsid may occur during genome packaging, permitting larger than unit length genomes to be incorporated (FRASER 1986a). Infectious ECVs are released by budding from the virus-modified cell surface. The cell membrane-derived envelope of these budded virions has peplomeric extensions of a viral encoded glycoprotein, gp64, that is a major neutralizing antigen and is apparently involved in adsorption of the virions to host cell surfaces (VOLKMAN et al. 1984).

Since the functions of nucleocapsid assembly and budding of infectious ECV are necessary for survival of the virus both in vivo as well as in vitro, they are sometimes referred to as essential functions (FRASER 1986b). In contrast, functions carried out in the second phase are necessary for survival of the virus in vivo, but not in vitro, and are considered to be nonessential or conditionally essential.

The second phase becomes apparent at about 20h post infection (p.i.) and continues until the cells expire. Nucleocapsids remaining within the nucleus at the beginning of the second phase are sequestered in de-novo-synthesized envelopes and then encapsulated within OBs (HUGHES 1972; STOLTZ et al. 1973; FRASER 1986a). The OBs are formed by the assembly of a paracrystalline matrix composed of a single protein called polyhedrin (SUMMERS and SMITH 1976). The encapsulation of virions in OBs protects the virus during desiccation, and helps stabilize the virus against extremes of heat and cold. Upon ingestion by a suitable host insect, the OB matrix is dissolved in the basic pH of the insect gut juices, and the embedded virions are released to initiate infection of the midgut epithelial cells (see FAULKNER 1981 for a review).

In a broad sense, the second phase of NPV replication accomplishes functions that are common among many insect viruses that infect the larval stages of insects that undergo complete metamorphosis. This phase can be likened to encapsulation in prokaryotes or sporulation in lower eukaryotes, and serves to insure survival of the viruses for prolonged periods within soil or on leaf surfaces. Similar strategies for environmental survival are employed by the closely related granulosis viruses (GV; Baculoviridae Subgroup B; MATTHEWS 1982), the insect-pathogenic poxviruses (entomopoxviruses; ARIF 1984) and the insect-pathogenic cytoplasmic polyhedrosis viruses (CPV; PAYNE and MERTENS 1983).

2.2 Expression of Baculovirus Genes

As with most viruses, the temporal regulation of baculovirus gene expression is a tightly controlled cascade initiating with the immediate early genes (alpha class), followed by the delayed early genes (beta class), both of which precede DNA replication (KELLY and LESCOTT 1981; CARSTENS et al. 1979; MILLER et al. 1983b). Alpha genes are first expressed following penetration and uncoating of

the virus in the cell nucleus (GUARINO and SUMMERS 1986). These genes do not require any previously coded viral proteins for expression (KELLY and LESCOTT 1981), since purified viral DNA is capable of initiating and completing the entire replication cycle (see below). The beta class of genes are dependent upon the alpha class products for expression (KELLY and LESCOTT 1981; MILLER et al. 1983b; GUARINO and SUMMERS 1986, 1987).

Following the initiation of genome replication (5–7 h p.i; CARSTENS et al. 1979), the late genes (gamma class) are expressed (CARSTENS et al. 1979; KELLY and LESCOTT 1981). These genes presumably encode functions related to virus structure and assembly. The expression of late genes corresponds with the release of ECV from the infected cells (CARSTENS et al. 1979; WOOD 1980; KELLY and LESCOTT 1981).

Most of the alpha, beta, and gamma gene products produced during the first phase of the replication cycle are essential to baculovirus viability. Such essential processes as viral specific gene activation, degradation of the host cell genome, replication of the viral genome, assembly of nucleocapsids, and transport and budding of virions from the cell surface are all mediated by these first-phase genes. Up to this point the baculovirus replication process superficially resembles that of many DNA animal viruses.

Baculovirus replication differs from other DNA animal virus groups in having a fourth temporal class of expressed genes. At about 20 h p.i. the release of ECV dramatically declines, expression of many alpha, beta, and gamma proteins is relatively reduced but not necessarily entirely eliminated (CARSTENS et al. 1979; WOOD 1980; GUARINO and SUMMERS 1986), and the delta class proteins predominate (CARSTENS et al. 1979; WOOD 1980; KELLY and LESCOTT 1981). The delta genes comprise the second phase of the baculovirus replication cycle. Among the products generated in AcMNPV infected cells during this second phase are those proteins involved in the construction of nuclear localized de novo envelopes, the 29-kDa polyhedrin protein that forms the paracrystalline matrix of the OBs, the 10 K protein whose function is unclear (SMITH et al. 1983a; VLAK et al. 1988), and the 34 K protein which is a component of the OB envelope (WHITT and MANNING 1988). The temporally regulated cascade of gene activation insures that maximal expression of foreign genes under control of the very late polyhedrin gene promoter, or other delta gene promoters, will not begin until well after virus replication is completed.

As mentioned before, these very late genes are considered nonessential or conditionally essential, and are superfluous to effective replication of the virus in cell cultures. However, mutations that abolish OB formation severely limit the replication potential of the virus under natural conditions and the virus cannot be maintained in the natural host unless infectious OBs are formed. In this respect the use of the polyhedrin substitution vector effectively introduces a form of biological containment for the baculovirus expression vectors.

2.3 Polyhedrin and Other Late Genes

Because of its relative abundance during NPV infections and its importance in the natural survival of the virus, polyhedrin was the first baculovirus protein to be identified and studied in detail. The conditionally essential nature of the polyhedrin gene made it an ideal first candidate for genetic engineering in the NPV system.

Polyhedrins of different baculoviruses are biochemically similar. The proteins isolated from both MNPVs and SNPVs are similar in size, isoelectric points, solubility properties, and immunoreactivity (see VLAK and ROHRMANN 1985 for a comprehensive review). Polyhedrins are readily solubilized at a pH of 9.5–10.5 or greater. All polyhedrins sequenced to date have a calculated molecular weight between 28 and 29 kDa, pI between 5.3 and 6.5, and are immunologically cross-reactive.

The polyhedrin proteins of NPVs are abundantly expressed at late times during infection. Polyhedrin is estimated to be as much as 25% of total infected cell protein (PENNOCK et al. 1984) and up to 50% of the stainable protein expressed during the second phase of replication (LUCKOW and SUMMERS 1988; MILLER 1988). The protein accumulates to estimated levels of 1 mg per 1–2 × 10^6 cells (LUCKOW and SUMMERS 1988), principally in the form of 50 to 100 OB particles per infected cell. These OBs are the predominant structure of wild-type infected cells (2–5 μm in diameter) and can be observed easily with a light microscope. Moreover, polyhedrins are one of the very few viral proteins that are produced throughout the entire second phase of NPV infections, permitting accumulation of the protein over an extended period (usually 48 h or more).

Many early studies of baculovirus replication suggested the nonessential nature of polyhedrin. Electron-microscopic observations of baculovirus maturation had always demonstrated that OBs and related second-phase structures form well after nucleocapsid assembly and ECV release (KNUDSON and HARRAP 1976; ADAMS et al. 1977). Polyhedrin synthesis in AcMNPV-infected cell cultures always became obvious from 20 h p.i. and reached a maximum between 36 and 48 h p.i. In contrast, infectious ECV release was detected by 8 h p.i. and became maximal around 18 h p.i. Ultimately, the nonessential nature of polyhedrin was proven by experimental elimination of the polyhedrin gene through in vitro mutagenesis and gene transfer (SMITH et al. 1983b).

The polyhedrin genes from a number of viruses have been sequenced in entirety (HOOFT VAN IDDEKINGE et al. 1983; IATROU et al. 1985; LEISY et al. 1986a). The size and structure of polyhedrin genes is quite similar, and there is a 5′ untranslated A + T rich leader sequence that is strikingly similar among all polyhedrin genes and begins with a 12–bp consensus sequence surrounding the mRNA start site (ROHRMANN 1986; HOWARD et al. 1986).

Another major late protein in AcMNPV-infected cells is the p10 protein. The exact function of this protein is still in doubt, but it appears to be involved in a nonessential way in the assembly or structuring of the occlusion body envelope (QUANT-RUSSELL et al. 1987; VAN DER WILK et al. 1987). Recent studies

have demonstrated that p10 is present as a component of the fibrous material (FM), a nuclear inclusion present at late times in infection and involved in formation of the OB envelope (VLAK et al. 1988). Deletion of the p10 coding region does not abolish FM formation (WILLIAMS et al. 1989), nor does it inhibit OB envelope formation (VLAK et al. 1988). Fusion of p10 with *lacZ* abolishes OB envelope formation, rendering the OBs more labile to solubilization (VLAK et al. 1988). In addition, the p10/*lacZ* fusion protein can be localized to the FM inclusion, but the exact function of p10 in development of FM or OB envelopes is unknown (VLAK et al. 1988).

3 Engineering Recombinant Baculoviruses

The potential utility of the polyhedrin gene to effect high-level expression of foreign genes in baculovirus-infected insect cells was recognized independently by several investigators (SMITH et al. 1983b; MILLER et al. 1983a; MAEDA et al. 1985).

In the first report of successful application of the baculovirus as an expression vector (SMITH et al. 1983c), the recombinant viruses incapable of producing OBs were distinguished from wild-type virus by a simple plaque assay in a manner analogous to the previously established detection methods for FP mutants (FRASER and HINK 1982). Baculovirus plaques are microscopic, and best viewed with a dissecting microscope at between 20 × and 70 × magnification. Illumination of the infected cell monolayers with obliquely directed light causes an opalescent appearance in wild-type virus plaques due to the presence of numerous refractive OBs in each infected cell. Those plaques lacking OBs (OB negative), or with significant reductions in the number of OBs per infected cell (i.e., FP mutants) are discernably less opalescent.

An alternative strategy was to construct *lacZ*-gene fusions at the natural *Bam*HI (+ 171 bp) within the polyhedrin coding region (PENNOCK et al. 1984). Co-transfections of recombinant plasmid and wild-type viral DNAs resulted in recombinant viruses that were selected based upon their ability to form blue plaques in the presence of the colorimetric indicator, X-gal, and their inability to form OBs.

The capacity for production of foreign genes in infected insect larvae was demonstrated by MAEDA et al. (1985). In this study, the polyhedrin coding region of *Bombyx mori* MNPV (BmMNPV) was localized using cDNAs prepared from mRNAs isolated from the fat bodies of infected silkworm larvae. The availability of the BM-N established cell line allowed propagation and manipulation of the virus to generate recombinants. In this case, however, the expression of the polyhedrin-controlled gene product was demonstrably more efficient in the larval system than in cell cultures.

Another major late protein gene of AcMNPV, p10, has been manipulated in a manner similar to the polyhedrin gene (VLAK et al. 1988). The gene was

localized to the *Eco*RI-P fragment by hybridization of abundantly expressed late mRNAs to viral gene fragments (SMITH et al. 1983a). Subsequent sequencing of the *Eco*RI-P fragment positioned the gene and it's promoter (KUZIO et al. 1984). A sequence similar to the consensus 5′ mRNA start site of polyhedrins is located upstream of the p10 gene as well (KUZIO et al. 1984; ROHRMANN 1986), suggesting this is a common recognition sequence for hyperexpression of late genes. Since this gene does not provide a selective phenotype, *lac*Z fusions were performed at the unique *Bgl*II site at + 153 bp, and recombinants were selected for blue-plaque morphology (VLAK et al. 1988). The *lac*Z recombinants were replication competent, as expected for a nonessential late gene, and were capable of forming OBs as well.

Engineering the p10 region for expression of genes necessarily requires either selection of clear plaques following cotransfection of a p10/*lac*Z recombinant with the p10 expression vector construct, or construction of transfer plasmids having both *lac*Z and the gene of choice under control of a hyperexpressed promoter. Such double-promoter expression vectors have been constructed and have proved effective (see below).

Promoters for other late genes that are either essential or nonessential may be utilized for expression vector construction. One example of the use of an essential gene promoter is the study of HILL-PERKINS and POSSEE (1990). In this instance they utilized the promoter for the major core protein gene (WILSON et al. 1987), a delta class gene product that seems to be associated with the packaging of viral DNA (TWEETEN et al. 1980; BUD and KELLEY 1980). The strategy involved duplication of the promoter for the core protein gene next to the *lac*Z gene inside the polyhedrin gene region. Substitution of the constructed gene for the nonessential polyhedrin gene generated virus expressing *lac*Z maximally between 8 h and 18 h p.i., as would be expected for a delta class promoter (HILL-PERKINS and POSSEE 1990). These authors point out that the use of promoters from earlier temporal classes may have some advantages for production of proteins requiring extensive post-translational modifications, or to express insect-specific toxins and hormones at earlier times in the infected insect than with the p10 or polyhedrin promoters.

3.1 The Polyhedrin Promoter

Examination of the 5′ regions from a number of polyhedrins has led to some interesting observations relative to baculovirus late gene promoter structure and sequences necessary for hyperexpression of polyhedrins and p10 proteins. The first reports of the polyhedrin gene sequences had indicated the presence of apparent TATA- and CAT-like promoter signals (HOOFT VAN IDDEKINGE et al. 1983; MAEDA et al. 1985; IATROU et al. 1985). However, the comparative analysis of upstream flanking sequences for a number of polyhedrin genes by ROHRMANN (1986) demonstrated little conservation of sequence and position of the TATA- and CAT-like regions. Instead, there was remarkable similarity in the length

and AT content of the 5′-nontranslated leader region. In addition, the comparison identified a consensus sequence surrounding the mRNA start site at nucleotide −49 in a number of polyhedrin genes (LEISY et al. 1986b). This consensus sequence, between nucleotide −43 and −54 (5′AATAAGTATTTT3′) is apparently essential for high level expression of the polyhedrin gene.

Several sequences within the nontranslated leader are clearly involved in optimal expression of the gene. MATSUURA et al. (1987) demonstrated that deletions in the leader region past −14 from the ATG start codon adversely affect the expression of genes fused to the polyhedrin promoter, while deletion of the entire coding region and 3′ flanking sequences does not appreciably alter their expression. POSSEE and HOWARD (1987) demonstrated that deletions in the 5′ flanking region from −69 to −92 did not affect levels of *lacZ* production by polyhedrin/*lacZ* recombinant viruses. However, deletion of the region from −47 to −56 (encompassing the transcriptional start site and the Rohrmann consensus sequence) resulted in significantly reduced levels of *lacZ*-specific transcript. They described the functional limits of the polyhedrin promoter as between −49 and −69. Similarly, RANKIN et al. (1988) demonstrated that removal of the region from −1 through to the *Eco*RV site at −92 bp reduces the expression of the chloramphenicol acetyl transferase (*CAT*) gene 1000-fold in a transient expression assay. Interestingly, positioning of this leader-containing region fused to the *CAT* gene in reverse orientation with respect to the flanking viral sequences increased the levels of *CAT* activity twofold. Successive substitution of a 10-bp *Hin*dIII linker for sequences within the 5′ flanking region from −83 to −1 was used to assess the relative importance of leader sequences on promoter function. The most dramatic reductions in *CAT* expression (nearly 1000-fold) were obtained with substitutions in the region from −42 to −60, once again demonstrating the importance of the consensus 5′ mRNA initiation signal in polyhedrin gene expression. OOI et al. (1989) suggested the importance of the nontranslated leader in optimal transcriptional initiation.

A sequence similar to the consensus 5′ start site of polyhedrins is located upstream of the p10 gene as well (KUZIO et al. 1984; ROHRMANN 1986), suggesting this is a common recognition sequence for hyperexpression of very late genes.

3.2 Available Polyhedrin-Based Baculovirus Transplacement Vectors

The baculovirus expression vector systems now available include those derived from AcMNPV, BmMNPV, and HzSNPV. No expression vectors are yet available for the other major subgroup of baculoviruses, the granulosis viruses, owing to a lack of suitable in vitro systems for their propagation and manipulation. The AcMNPV-infected *Spodoptera frugiperda* cell line system (SMITH et al. 1983c; PENNOCK et al. 1984; SUMMERS and SMITH 1987) has received the most attention due to the relative ease of handling, and the ready availability of the cell line, media, and virus. Several vector constructs are currently available

for the manipulation of the AcMNPV virus polyhedrin gene. The reader is referred to other recent reviews for a more complete description of available vectors and their activities (LUCKOW and SUMMERS 1988; LUCKOW 1991).

To date, the pAc373 construct has been the vector used most often. This vector is superior to pAc380 for expression of human interleukin-2 (IL-2; SMITH et al. 1985), probably because it retains the consensus transcription initiation signal and much of the 5′ leader sequence of the polyhedrin gene. The pAc373 vector contains a deletion from the *Bam*HI site (+171) of the wild-type AcMNPV polyhedrin sequence, through the ATG start codon, and 5′ untranslated leader sequence, to −8 from the polyhedrin messenger RNA cap site. Similar vectors have also been constructed by MATSUURA et al. (1986). The insertion of a *Bam*HI linker (pAcRP6) or a multiple cloning site (pAc373) allows insertion of genes in the untranslated leader region. In both cases, the natural *Bam*HI site at +171 is fused to the *Bam*HI site of the linker region to complete the constructions.

Similar manipulations resulted in the construction of the pAc610 vector, also used by several investigators. This vector differs from pAc373 in having a more extensive inserted polylinker sequence after nucleotide −7, and a more extensive deletion of the polyhedrin coding domain to nucleotide +670 near the terminus of the polyhedrin gene (LCUKOW and SUMMERS 1988). The pAcRP18 and pAcYM1 (MATSUURA et al. 1987) vectors contain a *Bam*HI linker fused at positions −1 and +1, respectively, pEV55 (MILLER et al. 1986) is constructed with a polylinker at position +1. pEVmod eliminates redundant sites in pEV55 at the pUC8/AcMNPV sequence junctions (WANG et al. 1991), and pEVmXIV substitutes a modified polyhedrin promoter, P_{XIV} (RANKIN et al. 1988; OOI et al. 1989) for the wild-type polyhedrin promoter (WANG et al. 1991). All of these vectors leave all, or nearly all, of the nontranslated leader sequence intact. Both fused and nonfused expression vectors are available for BmMNPV as well (S. Maeda, personal communication).

The pAcCL29-1 and pAcCL29-8 vectors are derivatives of pAcYM1 that contain an M13 origin of replication which permits production of single-stranded DNA in the presence of a helper phage (LIVINGSTON and JONES 1989). These vectors facilitate site-directed mutagenesis for analysis of expressed genes. A similar strategy was used by HASEMANN and CAPRA (1990) by incorporating the F1 origin of replication into pAc360-derived transfer vectors containing murine immunoglobulin heavy and light chain regions.

From the first descriptions of expression vectors it was noted that in many cases the expression of genes fused in-frame with the polyhedrin coding sequences is much greater than those placed near the start of transcription, or even near the start of translation. Levels of both human beta-interferon and bacterial beta-galactosidase in AcMNPV were quite high if expressed as in-frame fusions with the polyhedrin coding sequences rather than as nonfused proteins (SMITH et al. 1983c; PENNOCK et al. 1984). LUCKOW and SUMMERS (1988) indicated that in-frame fusions of 30 amino acids or more of polyhedrin to several protein genes increased the levels of expressed protein and RNA. Apparently sequences

in the 5′ amino-terminal region of the polyhedrin coding domain are also involved in optimal expression of the polyhedrin gene.

More recently constructed vectors, such as the pVL941 vector (LANFORD 1988) or its derivatives pVL1392 and pVL1393 (mentioned in LUCKOW 1991), contain an alteration of the polyhedrin ATG start codon to ATT, allowing genes to be inserted with their own ATG start codons at a *Bam*HI site downstream of the normal polyhedrin translation initiation sites. The pVL941 vector provided twofold higher levels of SV40 large T antigen expression than the pAc373 vector (LANFORD 1988).

A similar vector, p36C, was constructed by mutagenesis of the ATG start codon to ATC in the polyhedrin fusion vector pAc360 (PAGE 1989). Genes are inserted at the retained *Bam*HI site at +33, and levels of expressed protein product are reportedly 5 times greater than with pAc373.

There is ample evidence that the 3′ terminal regions of the polyhedrin gene have little, if any, effect on expression of the gene. Deletions of this region failed to introduce any appreciable alterations in levels of expressed RNA or protein (MATSUURA et al. 1987; POSSEE and HOWARD 1987).

The relative levels of expression obtained with each of these vectors depends to a great extent on the gene being expressed. While optimal expression of similar genes is effected when all of the 5′ leader and some polyhedrin coding domain are intact (e.g. LANFORD 1988), the actual amount of a given protein product generated is most dependent on the gene being expressed (LUCKOW and SUMMERS 1988).

Recently, a vector based upon a synthetic promoter, P_{syn} (WANG et al. 1991), has been constructed from comparisons of polyhedrin and p10 (see below) promoter regions. Analyses of *CAT* gene expression have demonstrated that this promoter is less efficient than the wild-type polyhedrin promoter, but can be used at numerous alternative regions in the virus genome without duplicating existing baculovirus sequences, thus avoiding potential instability (WANG et al. 1991).

3.3 Available p10-Based Baculovirus Transplacement Vectors

Expression vectors utilizing the promoter for p10 have been constructed and demonstrated to be effective for foreign gene expression. The overall selection strategy relies upon the prior construction of a p10-*lacZ* recombinant virus to serve as recipient. p10 expression vector recombinants generate white plaques from these p10-*lacZ* recipient viruses.

The vector pAcUW1 provides a unique *Bgl*II site at position +1 in the p10 gene (WEYER et al. 1990) and essentially mimics the promoter structure of pAcYM1. The effectiveness of this vector was demonstrated using both the *lacZ* gene and the polyhedrin gene. The p10-*lacZ* fusion gene was also used as a selectable marker for polyhedrin-based expression vectors. The pAcUW2 constructs (WEYER et al. 1990) allow insertion of genes under control of the p10

promoter and just upstream of a functional polyhedrin gene. Recombinant virus generated from this sort of vector would be capable of forming OBs while expressing the gene of choice. Such constructs could have advantages for scale-up in insect larvae or for genetically improved biological insecticides.

The pAcAS2 vector (VLAK et al. 1990) was constructed with a pUC19 multiple cloning site at position +1 in the p10 gene. Subsequent addition of a *Drosophila* hsp70 promoter-driven *lacZ* gene to produce the vector pAcAS3 provides a selectable marker for detecting recombinant expression viruses. The utility of the pAcAS3 vector was demonstrated by expression of cauliflower mosaic virus gene 1 (VLAK et al. 1990).

3.4 Practical Considerations

Among the advantages of the baculovirus system are the relative ease of handling of both the virus and cell cultures and the speed with which one can engineer and isolate recombinants for analysis. It is not unreasonable to have plaque purified recombinant viruses for analysis within 6 weeks from successful cloning of the gene to be expressed (either an intronless genomic or cDNA copy of the gene is advised; see below).

The basic technology of gene replacement or allelic replacement is employed for genetic engineering of the baculovirus genome. Essentially, this involves the transfection of viral and recombinant plasmid DNAs into susceptible insect cell cultures and the identification of recombinants based on the absence of the large intranuclear OB inclusions. Most of the procedures commonly employed in working with baculoviruses have been assembled as a manual by SUMMERS and SMITH (1987).

The transfection of lepidopteran insect cells with viral or plasmid DNAs is most conveniently accomplished using the $CaPO_4$ coprecipitation procedure originally developed for mammalian cells (GRAHAM and VAN DER EB 1973; BURAND et al. 1980; POTTER and MILLER 1980a; CARSTENS et al. 1980). Modifications of this precipitation procedure have been employed on occasion, but are generally not as reliable. The modification typically employed for transfection of *Drosophila* cell lines (DINOCERA and DAWID 1983) is much less effective for lepidopteran cells. Alternative transfection procedures such as the polybrene method (KAWAI and NISHIZAWA 1984) are less effective than the $CaPO_4$ method for lepidopteran cells and baculorius DNAs. Lipofection (BRL) has also been used (SHIU et al. 1991), and in our hands this procedure is effective but has not represented a significant improvement over $CaPO_4$ technique (M. J. Fraser, unpublished). Electroporation is very effective for a *S. frugiperda* cells in particular (MANN and KING 1989) and other lepidopteran cell lines in general (M. J. Fraser, unpublished), but may be inconvenient for general use.

In general, the relative efficiency of the $CaPO_4$ transfection procedure is related to a number of factors influencing the precipitation reaction (GRAHAM et al. 1980). In addition to proper formation of the precipitate, the most important

factors include the quality of the viral and plasmid DNAs and the cell line employed. For the generation of baculovirus recombinants, the greatest efficiencies and most consistent results are obtained with CsCl gradient purified viral and plasmid DNAs. Dialysis of the DNAs against 0.1 × SSC is recommended over ethanol precipitation as a final step in the isolation (CORSARO and FRASER 1989).

The IPLB-SF21AE cell line (VAUGHN et al. 1977) and derivatives (i.e., Sf-9; SUMMERS and SMITH 1987) is an ideal recipient cell line for genetic manipulations of AcMNPV by transfection. In contrast, the TN-368 (HINK 1970) cell line is not as receptive to transfection with the same preparations of AcMNPV. The UND-K derivative of the IPLB-HZ 1075 cell line (CORSARO and FRASER 1987b) is suitable for manipulation of HzSNPV while the parent cell line, IPLB-HZ 1075 and several other cloned derivatives are less receptive (CORSARO et al. 1989). The BM-N cell line (MAEDA et al. 1985) is suitable for genetic manipulations with BmMNPV.

Once the viral and plasmid DNAs are transfected into the recipient cell line, recombination between viral sequences on the replacement vector and the same regions of the viral genome takes place (SMITH et al. 1983b, c; PENNOCK et al. 1984; MAEDA et al. 1985). This recombination event is presumably mediated by cellular factors, but is also influenced by the extent of unmodified flanking viral sequences in the transplacement vector (for a review see LUCKOW and SUMMERS 1988) Most polyhedrin-based vectors currently employed retain a minimum of 7 kb flanking viral DNA. With such vectors, the recombination efficiency is of the order of 0.1%–5%.

The quality of the viral DNA is also critical in deriving recombinant viruses. We have noted for some time that supercoiled viral DNA is 5 times more infectious in transfections of *S. frugiperda* cell cultures (CORSARO and FRASER 1989a), but gives a reduced yield of recombinant virus compared to the nicked circular or linear forms (unpublished observations). KITTS et al. (1990) took advantage of this fact in engineering a virus, AcRP6-SC, that contains a unique *Bsu*36I site within the polyhedrin gene. Linearization of the viral DNA prior to cotransfection with the polyhedrin transfer vector containing the gene to be expressed decreased the overall infectivity of the transfected viral DNA, but increased the relative yield of recombinant virus to between 6% and 32% of the progeny virus. Similar results were obtained for linearization of viral DNA at the p10 locus using p10 gene transfer vectors. These results suggest that only circularized viral DNA molecules may replicate, and that addition of the appropriate transfer vector provides a means for selective repair within a given locus by recombination with the digested viral DNA. This effectively increases the relative proportion of progeny virus that are recombinant.

GOSWAMI and GLAZER (1991) report success using plasmid DNA purified by passage through Quiagen columns (Quiagen, Studio City, CA) to transfect Sf-9 cells that had been previously infected with wild-type AcMNPV. The advantage of this approach is the elimination of lengthy procedures for preparation of viral and plasmid DNAs. However, direct comparisons with alternative

preparations and estimates of relative efficiency of recombinant virus recovery were not made.

3.5 Detecting OB-Negative Recombinant Plaques

Selection of recombinant virus can be accomplished in a number of ways. The most commonly employed technique for distinguishing recombinants takes advantage of the reduced refractivity of plaques that produce fewer OBs than the wild-type virus. This detection method had been previously perfected through analysis of the spontaneous FP mutants of baculoviruses (FRASER and HINK 1982). The ability to visually detect the recombinant plaques depends upon optimization of the plaque assay methodology. The fact that some researchers experience difficulty detecting recombinants in this way indicates these factors are not trivial.

The initial seeding cell density, overlay formulation, and incubation conditions can all influence the detection of OB-negative plaques (FRASER and HINK 1982). Because the virus spreads relatively slowly in the monolayer, the cells must be seeded at a density allowing growth of the monolayer for several days. Early death of the monolayer will preclude plaque formation or hamper detection of recombinants. A low initial cell density will not yield a dense enough monolayer for sufficient localized cell death to permit detection of recombinant plaques. The optimal cell seeding density differs for a given cell line, and is largely dependent upon the cell doubling time. If detection of OB-negative plaques is difficult, plaque assays of an OB-negative mutant control virus should be performed with varying cell seeding densities to optimize conditions for detection. We have found optimal plaque formation with *S. frugiperda* cells occurs with seeding densities of between 1.1 and 1.25×10^3 cells per mm^2 of available plate surface.

Staining viable cells for 15–20 min with a small volume of sterile 0.01%–0.05% neutral red in PBS added over the agarose overlay and viewing the infected monolayer 4–8 h later enhances the detection of OB negative plaques significantly. The alternative strategy of adding neutral red directly to the overlay mixture (KNUDSON 1979) has not been as successful in our hands.

Checking the putative recombinant plaques at 200X magnification with an inverted microscope is highly recommended prior to picking. Potential false positives, such as the spontaneous FP mutants that also generate plaques of reduced refractivity, can occasionally be found in preparations of wild-type virus. These FP mutants can be distinguished from recombinants by the presence of at least some OBs in infected cells of the plaque (see FRASER 1986b, for review). Since these mutants can be amplified upon continued propagation of the virus in cell cultures, baculovirus DNAs used for transfections to generate recombinants should be prepared from plaque-purified stocks of wild-type virus that has undergone fewer than five passages in cell culture, or from OBs purified from peroral-infected insect larvae.

Several cycles of plaque purification are recommended to insure purity of the recombinant virus. Multiple potential recombinants should be isolated for analysis at this stage. As a final check, the putative recombinants should be analyzed with restriction enzymes, and possibly Southern hybridization, to insure the desired DNA fragment is present. As with any virus, the baculovirus genome is a dynamic entity (KUMAR and MILLER 1987) subject to rearrangements (BURAND and SUMMERS 1982), insertional mutagenesis by host cell sequences (POTTER and MILLER 1980b; FRASER et al. 1983; MILLER and MILLER 1982) and other less apparent types of mutations. Checking recombinants by restriction enzyme analysis and other means prior to amplification insures at least that major alterations of the viral genome have not taken place.

3.6 Alternative Selection Schemes

Alternative detection methods have occasionally been employed with varying success. The ability to adsorb red blood cells can help identify recombinant virus-infected cells that are producing viral hemagglutinins such as the influenza or parainfluenza envelope glycoproteins (KURODA et al. 1986, 1987; VAN WYKE COELINGH et al. 1987).

Limiting dilution and DNA dot-blot hybridization has been employed with success to detect and purify recombinant virus (FUNG et al. 1988; PEN et al. 1989; GOSWAMI and GLAZER 1990). This method allows selective amplification of recombinant virus, avoids potential selection of false-positive plaques, and works well with even low recombination frequencies. Hybridization plaque lift assays may also be employed (VILLAREAL and BERG 1977; MILLER et al. 1986; SUMMERS and SMITH 1987; JEANG et al. 1987b), but these assays meet with varying success, and additional manipulations will be necessary to optimize conditions.

Newer vectors are available that take advantage of the selective *lacZ* marker gene in plaque assays. These vectors rely on alternative promoters to express the *lacZ* gene, while the polyhedrin promoter is reserved for expression of the gene of choice. Blue viruses are easily detected after addition of the colorimetric substrate X-gal to the agarose overlay (PENNOCK et al. 1984). One example of this approach is the pJV(NheI) vector (VIALARD et al. 1990), a derivative of pAc373 that contains a p10 promoter expressing beta-galactosidase in opposite orientation next to a polyhedrin promoter region reconstituted to include sequences from the polyhedrin gene up to +33, with the normal ATG start codon altered to ATT. VIALARD et al. (1990) report that at least 70% of *lacZ*-positive viruses also express the gene of choice.

Another such vector, pAcDZ1, constructed by ZUIDEMA et al. (1990) utilizes a chimeric gene formed from the *Drosophila melanogaster* 70 K heat shock gene promoter (hsp70), the beta-galactosidase protein coding domain, and the SV40 early region termination signals. In this case, the polyhedrin gene and the chimeric *lacZ* gene are opposed, with the SV40 termination signals separating them. The levels of expressed protein product obtained with pAcDZ1 were

similar to those obtained with the conventional polyhedrin-based expression vectors (ZUIDEMA et al. 1990). A similar strategy was employed for expression vectors based on the p10 promoter (VLAK et al. 1990).

LacZ-selectable vectors are also commercially available from Invitrogen Corporation (San Diego, CA) as part of the MaxBac^R Baculovirus Expression System kit. The only potential problem with the *lacZ* co-expression approach may be that the increased size of these transfer vectors can make cloning certain genes for expression difficult.

4 Expression of Foreign Genes in Baculovirus-Infected Insect Cells

The attractiveness of the baculovirus-infected insect cell expression system rests on the fact that it allows for extremely efficient expression of protein products (averaging 1–10 μg per 10^6 cells) in a higher eukaryote cell system. Maximal expression of the protein occurs after the essential phase of virus replication, potentially allowing expression of gene products that may be cytotoxic (MILLER 1988). The expression system itself provides a level of biological containment (MAEDA 1987; MILLER 1988) because introduction of foreign genes into the polyhedrin promoter abolishes a function necessary for survival of the virus under natural conditions.

The baculovirus is capable of accommodating a large excess of sequences without appreciable effect on replication efficiency. The largest inserted fragment to date is approximately 10 kb (CARBONELL et al. 1985). Larger insertions up to 15 kb have been observed, based upon analyses of spontaneous mutations (FRASER 1986a). However, the stability of larger inserts still needs to be explored. It is possible that tandemly duplicated sequences or sequences flanked by inverted repeats will be unstable in this recombination competent system.

Another attractive feature of the system is the possibility of altering the viral genome at several locations due to the presence of multiple nonessential genes and intergenic mutable regions. As the location of mutable regions and additional late genes becomes known, more areas will be available for manipulation. This should permit incorporation of several highly expressed genes at multiple locations throughout the genome. In this regard, the manipulations of EMERY and BISHOP (1987) demonstrated the feasibility of incorporating more than one polyhedrin promoter in a single virus. Both proteins, the native polyhedrin protein and the recombinant lymphocytic choriomeningitis virus N protein, accumulated to substantial levels in the infected cells. However, the relative amount of polyhedrin produced by the recombinant virus was somewhat reduced compared to the control AcMNPV. Whether these reduced levels were significant was not determined.

Coinfection of insect cells with a number of different recombinant viruses is an alternative strategy that has proven effective for simultaneous expression of multiple gene products. St Angelo et al. (1987) demonstrated the effective coexpression of three influenza polymerase subunit genes (PA, PB1, and PB2) in Sf-9 cells coinfected with three recombinant viruses each expressing an individual gene. Expression of these genes occurs within the same cell, since protein complexes are formed between two of the subunits (PB1 and PB2). Immunoglobulin heterodimers could be formed by coinfecting Sf-9 cells with two recombinant viruses carrying either heavy- or light-chain murine immunoglobulin genes (Hasemann and Capra 1990).

The expression of multiple genes by coinfection offers opportunities for studying the interaction of overexpressed proteins in a eukaryotic cell environment devoid of significant background cellular protein synthesis. Expression of multiple mammalian genes in insect cells provides an environment potentially free of interfering proteins. Coexpression of the mouse p53 protein and the SV40 large T antigen in recombinant-infected insect cells resulted in typical complex formation between the two recombinant proteins, similar to that which would occur in SV40-infected mouse cells (O'Reilly and Miller 1988). Co-infection with recombinants expressing $pp90^{rsk}$ and $pp60^{v-src}$ resulted in activation of the serine-specific protein kinase activity of *rsk* through tyrosine phosphorylation by *src* (Vik et al. 1990).

The overexpression of eukaryotic gene products in insect cell lines provides several advantages over conventional prokaryotic expression systems. Post-translational modifications such as signal peptide cleavage, N-linked and O-linked glycosylation, additional proteolytic cleavages (although perhaps not identical to those of mammalian cells), and proper cellular compartmentalization of protein products (i.e., membrane localization, extracellular secretion, cytosolic localization, nuclear localization) all occur in baculovirus-infected insect cells. Both intrachain and interchain disulfide bridge formation have been observed (Geise et al. 1989). These is now ample evidence for RNA splicing as well (Chisholm and Henner 1988; Jeang et al. 1987a; Iatrou et al. 1989), although the preferential splicing of sites other than those preferred in mammalian cells has been observed (Jeang et al. 1987a).

Some advantages have been noted for baculovirus-expressed proteins compared to expression of the same proteins in bacteria. Insoluble protein aggregates that frequently form upon overexpression of genes in prokaryotes may not occur during expression with baculoviruses. Jeang et al. (1987b) point out that the p40x protein of HTLV-I forms aggregates when expressed in *Escherichia coli*. In contrast, insoluble aggregates are not formed by the same protein expressed in the baculovirus expression vector system, even though each infected cell produced 50–100 times more protein than the bacterial system. Baculovirus-produced Rap1A protein was both soluble and biochemically active as opposed to the same protein produced in *E. coli* (Quilliam et al. 1990). Hseih et al. (1989) report 5 times greater levels of expression for rat liver Yb_1 glutathione S-transferase than in *E. coli*.

4.1 Proteolytic Cleavages in Baculovirus-Infected Insect Cells

There is ample evidence that signal peptides are correctly cleaved from a number of diverse membrane-bound or secreted proteins expressed in baculovirus infected insect cells. Amino-terminal analysis has confirmed correct signal peptide cleavage for the human alpha-interferon (MAEDA et al. 1985), human gastrin-releasing peptide (LEBACQ-VERHEYDEN et al. 1988), human IL-2 (SMITH et al. 1985), mouse IL-3 (MIYAJIMA et al. 1987), human glucocerebrosidase (MARTIN 1988), human T-cell immune activation gene *Act*-2 (LIPES et al. 1988), *Phaseolus vulgaris* beta-phaseolin (BUSTOS et al. 1988), Sindbis virus E1 envelope protein (OKER-BLOM and SUMMERS 1989), and immunoglobulin heavy- and light-chain proteins (HASEMANN and CAPRA 1990). Correct cleavage of signal peptides is inferred from activity assays, transport and secretion, and size analysis on SDS-polyacrylamide gels for a number of other proteins. Examples include human beta-interferon (SMITH et al. 1983c), human erythropoietin (WOJCHOWSKI et al. 1987), the influenza hemagglutinins (POSSEE 1986; KURODA et al. 1986), parainfluenza type 3 hemagglutinin-neuraminidase (VAN WYKE COELINGH et al. 1987), the HIV envelope glycoprotein gp160 (HU et al. 1987; RUSCHE et al. 1987; COCHRAN et al. 1987), *hst-1* transforming protein (MIYAGAWA et al. 1988), leech antistasin (HAN et al. 1989), G1 and G2 glycoproteins of Rift Valley fever virus (SCHMALJOHN et al. 1989), the S glycoprotein of bovine corona-virus (YOO et al. 1990), human granulocyte-macrophage colony stimulating factor (CHIOU and WU 1990), and the alpha subunit of human chorionic gonadotropin (NAKHAI et al. 1991). The insect cells did not recognize and cleave a signal peptide for a bacterial protein, the *Bacillus anthracis* protective antigen (IACONO-CONNORS et al. 1990).

Some types of post-translational proteolytic and protein modifying processes are apparently lacking or different in insect cells. Detailed comparisons of the processing of human gastrin releasing peptide precursor in baculovirus-infected insect cells and a mammalian lung cancer cell line revealed significant differences in several proteolytic cleavages (LEBACQ-VERHEYDEN et al. 1988). No carboxy-peptidase B-like activity, trypsin-like endopeptidase activity, or peptidyl glycine alpha-amidating monooxygenase activity was detected following processing in Sf-9 cells. Instead, peptides that were similar in size to several of those present in the mammalian cell line were produced by proteases with previously undefined specificities.

WATHEN et al. (1989a) report that there are significant differences in the cleavage of the F_0 precursor glycoprotein of human respiratory syncytial virus (RSV) produced in insect cells. The extent of cleavage F_0 to F_1 and F_2 was markedly reduced compared to the vaccinia-expressed protein in Vero cells, and a secondary cleavage of the F_1 molecule was seen in the baculovirus-expressed protein. However, the proteins produced in insect cells were capable of inducing a neutralizing antibody response in cotton rats, although at lower levels than with protein from RSV-infected cells. Similarly, the F_0 glycoprotein of measles virus was only partially cleaved when expressed in insect cells,

suggesting these cells are deficient in this endoproteolytic activity, while monkey kidney cells cleave this protein efficiently (VIALARD et al. 1990). However, VIALARD et al. (1990) indicate that an insect cell line from *Trichoplusia ni* was able to cleave the F_0 more efficiently, confirming that there may be considerable variation in proteolytic activities among established lepidopteran cell lines. The baculovirus-expressed F_0 glycoprotein of humam parainfluenza type 3 was not cleaved at all into the F_1 and F_2 subunits in insect cells (RAY et al. 1989), but the recombinant protein was effective in inducing a protective immune response in hamsters.

Although the study of LEBACQ-VERHEYDEN et al. (1988) detected no trypsin-like endoprotease activity operating at the lysine residues in the sequence Gly-Lys-Lys-Ser, a trypsin-like endoprotease activity and a carboxypeptidase N activity were inferred from correct maturation of the influenza (fowl plague) virus hemagglutinin (KURODA et al. 1986, 1987). In this sutdy the majority of recombinant hemagglutinin produced in *S. frugiperda* cells was uncleaved precursor HA, but the cleaved products HA1 and HA2 were also apparent. The correct cleavage at the carboxy-terminal Arg and Gly residues the sequence Lys-Lys-Arg-Lys-Lys-Arg-Gly is essential for activation of the hemolytic fusion activity of the influenza hemagglutinin. Since such an activity could be isolated from homogenates derived from recombinant infected *S. frugiperda* cells, correct cleavage was inferred (KURODA et al. 1986, 1987).

This observation was believed significant because many other vertebrate glycoproteins have similar cleavage sites, and might be expected to be efficiently processed as well. However, no endoproteolytic cleavage of precursor HA to HA1 and HA2 was observed with the hemagglutinin of a human influenza virus (POSSEE 1986). Similarly, cleavage of the gp160 envelope glycoprotein of the HTLV-IIIB isolate of HIV to gp120 and gp41 was not detected by immunoblotting of total infected cell proteins separated on SDS-polyacrylamide gels (RUSCHE et al. 1987), or by immunoprecipitation of baculovirus-produced LAV gp160 (HU et al. 1987). Apparently certain trypsin-like recognition sites will be effectively cleaved while others will not. The exact reason for this discrepancy is not clear.

Finally, a 5′ terminal fragment encoding the C, M, and E structural proteins and the NS1 and NS2a nonstructural proteins of dengue virus 4 was expressed using the pAc373 vector (ZHANG et al. 1988). In this case, cotranslational processing of the polyprotein sequences resulted in apparently authentic NS1 and E glycoproteins, and such processing is believed to require the specific acitivity of cellular proteolytic enzymes. However, the levels of E and NS1 produced were only 25% of that generated in dengue virus-infected primate cells. Even so, the baculovirus-infected cell lysates were capable of inducing immunity to dengue virus in mice, apparently as a result of antibodies generated to the recombinant NS1 glycoprotein (ZHANG et al. 1988).

4.2 N-Linked Glycosylation and Fucosylation

Most glycoproteins that have been expressed in baculovirus-infected cells are immunologically active and, in some cases, have been effective in inducing protective immunity. However, the published reports on expressed glycoproteins reflect considerable variability concerning the exact nature and extent of glycosylation that can occur on various proteins. Simple size comparisons of glycoproteins produced in both baculovirus-infected insect cells and mammalian cells often seem to reveal differences in the extent of glyosylation (KURODA et al. 1986, 1987, 1990; POSSEE 1986; HU et al. 1987; RUSCHE et al. 1987; COCHRAN et al. 1987; STEINER et al. 1988; GREENFIELD et al. 1988; FURLONG et al. 1988; DOMINGO and TROWBRIDGE 1988; KRISHNA et al. 1989; BAILEY et al. 1989; GRABOWSKI et al. 1989; GEORGE et al. 1989; QUELLE et al. 1989; JOHNSON et al. 1989; OKER-BLOM et al. 1989; GERMANN et al. 1990; YOO et al. 1990; SANCHEZ-MARTINEZ and PELLETT 1991; VAN DRUNEN LITTLE-VAN DEN HURK et al. 1991). In most cases these discrepancies have been demonstrated, through further analyses, to result from differences in type and extent of glycosylation of the protein between insect cells and mammalian cells.

There are at least two reports in which baculovirus-produced glycoproteins are not glycosylated at all. Neither the human multidrug transporter P-glycoprotein (GERMANN et al. 1990) nor the recombinant extracellular domain of the human nerve growth factor (VISSAVAJJHALA and ROSS 1990) were glycosylated.

Tunicamycin treatment (SMITH et al. 1983c, 1985; BUSTOS et al. 1988; HASEMANN and CAPRA 1990; SANCHEZ-MARTINEZ and PELLETT 1991; JANSEN et al. 1991; NIIKURA et al. 1991a) and labeling with radiolabeled mannose (SMITH et al. 1983c, 1985; HU et al. 1987; COCHRAN et al. 1987; BAILEY et al. 1989) have both effectively demonstrated N-linked glycosylation. Enzymatic digestions have also been employed to characterize the glycosylation processes. N-linked glycosylation has been demonstrated in baculovirus-infected insect cells through the use of N-glycanase (RUSCHE et al. 1987; WOJCHOWSKI et al. 1987; MARTIN et al. 1988; GRABOWSKI et al. 1989; QUELLE et al. 1989; CHIOU and WU 1990), endoglycosidase-F (COCHRAN et al. 1987; DOMINGO and TROWBRIDGE 1988; WATHEN et al. 1989a, b; GERMANN et al. 1990; DESPRES et al. 1991) and Glycopeptidase-F (STEINER et al. 1988; JOHNSON et al. 1989; VAILARD et al. 1990). The presence of high mannose oligosaccharides is also indicated through the use of endoglycosidase-H (TARENTINO and MALEY 1974; MARTIN et al. 1988; WEBB et al. 1989; GRABOWSKI et al. 1989; DOMINGO and TROWBRIDGE 1988; GREENFIELD et al. 1988; JOHNSON et al. 1989; OKER-BLOM and SUMMERS 1989; FELLEISEN et al. 1990; DESPRES et al. 1991), and by adsorption of expressed glycoproteins to concanavalin A-Sepharose columns (STEINER et al. 1988; BUSTOS et al. 1988; QUELLE et al. 1989), and the trimming of high mannose residues in indicated by conversion of some glycoproteins to endoglycosidase-H resistance upon secretion (COCHRAN et al. 1987; JARVIS and SUMMERS 1989; JOHNSON 1989; SISSOM and ELLIS 1989; DESPRES et al. 1991). However, the extent of this processing is apparently reduced from that in mammalian cells (JARVIS and SUMMERS 1989).

JARVIS and SUMMERS (1989) also employed inhibitors of the mannose oligosaccharide processing pathway to identify the enzyme activities present in baculovirus infected Sf-9 insect cells. Both castanospermine and N-methyl-deoxynojirimycin apparently inhibited processing of high mannose residues, suggesting the presence of glucosidases I and/or II in Sf-9 cells. Analyses with inhibitors of mannosidase I and II activity were less convincing, but the presence of some mannosidase activity is strongly implied by conversion of glycoproteins to endoglycosidase-H resistance.

The complex class of N-linked oligosaccharides have not been generally observed for proteins produced in insect cells. Levels of galactosyl and sialyl transferases had been reported to be negligible in insect cells (BUTTERS et al. 1981) and terminal sialic acid residues are not generally observed in insect glycoproteins (KURODA et al. 1986, 1987, 1990).

There are, however, an increasing number of reports indicating that some proteins may undergo complex glycosylations. Several studies have produced baculovirus-expressed glycoproteins similar in size to their authentic counterparts (JARVIS and SUMMERS 1989; WHITEFLEET-SMITH et al. 1989; WEBB et al. 1989; RAY et al. 1989; KLAIBER et al. 1990; SCHMALJOHN et al. 1989, 1990; RODEWALD et al. 1990; KOENER and LEONG 1990; NAGY et al. 1990; SHIU et al. 1991; NIIKURA et al. 1991a; JANSEN et al. 1991). Since many of these authentic proteins are known to have complex oligosaccharides, the similarity in size on gel electrophoresis suggests similar patterns of glycosylation in the baculovirus-expressed proteins.

Fucosylation of N-linked oligosaccharides has also been recently confirmed. WATHEN et al. (1991) analyzed N-linked oligosaccharides with bovine epididymis alpha-fucosidase and detected the presence of a fucosylated trimannosyl structure. This confirms the primary observation by KURODA et al. (1990) implying the presence of fucosyltransferases in insect cells.

DAVIDSON et al. (1990) provided the first evidence of complex glycosylation of a protein expressed in insect cells. Anion-exchange liquid chromatography mapping of glycopeptidase F-released oligosaccharides demonstrated that approximately 40% of baculovirus-expressed human plasminogen released bisialo-biantennary complex-type carbohydrate. Subsequent analysis (DAVIDSON and CASTELLINO 1991) showed that the appearance of complex carbohydrates on recombinant human plasminogen was dependent on the time post infection. Early in the infection (0–20 h p.i.) approximately 96% of all oligosaccharides are of the high mannose type, while 92% of the those released from recombinant plasminogen between 60 and 90 h p.i. were of the comlex type. These results suggest that the normal glycosylation patterns of the insect cells may be altered in response to the presence of recombinant human plasminogen. Similar analyses of baculovirus-expressed plasminogen in the *Mamestra brassicae* insect cell line IZD-MB0503 revealed that while normal insect cell proteins may not contain N-linked complex oligosaccharides the glucosyltransferase activities required for assembly of complex oligosaccharides can be effected upon infection with the human plasminogen-expressing baculovirus recombinant (DAVIDSON and

CASTELLINO 1991). A possible explanation for the apparent inducibility of complex glycosylation in insect cells during baculovirus infection is that (alpha 1, 2)-D-mannosidase-like enzyme activity is relatively low in IPLB-SF21AE cells but is somehow stimulated upon infection with either wild-type AcMNPV or a plasminogen-expressing recombinant (DAVIDSON et al. 1991).

The apparent variability between reports of oligosaccharides processing in insect cells should not be unexpected. Patterns of N-linked glycosylation can vary with the cell line employed, culture media and supplements used, length of time post infection, and most importantly, the protein itself (PAREKH et al. 1989). Many of these variables differ between individual reports of glycoprotein expression in baculovirus-infected lepidopteran cells.

Not all cell types will perform glycosylation in the same way (PAREKH et al. 1989). The lepidopteran cell lines used for baculovirus-mediated expression vary among different laboratories. For example, many reports use the Sf-9 subclone of the IPLB-SF21AE cell line, while others use the parental SF21AE cells. At least one paper reports the use of a Sf158 subcloned cell line derived from an unspecified *S. frugiperda* cell line. KURODA et al. (1986, 1990) and SHIU et al. (1991) report a *S. frugiperda* cell line, but do not specify the source.

Given the fact that changes in the physiological condition can alter patterns of glycosylation (PAREKH et al. 1989) the media and culture conditions used for analysis of glycosylated patterns may influence the extent and timing of glycosylation in a given cell line. Media formulations employed for baculovirus-mediated expression range from TNM-FH with or without serum supplement, Grace's with or without serum, TC-100 with or without serum, EX-CELL 400 serum-free medium (JRH Biosciences, Lexana, KS), or other serum-free formulations (see below).

For any given combination of cell type, medium, culture conditions, time of harvesting, and protein expressed, the results are quite consistent. However, glycosylation patterns seen for a specified protein under a given set of conditions may not be generally seen for all glycoproteins under the same conditions. The patterns and timing of glycosylation are highly dependent upon the protein being expressed (PAREKH et al. 1989).

While most glycosylation events in insect cells may not be identical to those of mammalian cells, they are evidently sufficient to allow extracellular transport of secretory proteins and cells surface presentation of membrane glycoproteins. They also do not preclude formation of tertiary and quaternary structures essential for biological activity or antigenic potential (e.g., fowl plague virus HA protein, KURODA et al. 1986; parainfluenza virus type 3 HN protein, VAN WYKE COELINGH et al. 1987; hepatitis B virus S antigen 22 nm particle formation, KANG et al. 1987; SCULLY and KANG 1988; vesicular stomatitis virus G protein, BAILEY et al. 1989; Rift Valley fever virus G1 and G2 proteins, SCHMALJOHN et al. 1989; Newcastle disease virus HN protein, NIIKURA et al. 1991a).

The accumulated evidence suggests that baculovirus recombinant glycoproteins can be expected to be immunologically and functionally similar to the native proteins. In some cases they may even be superior to mammalian

cell devied protein in inducing an immune response (e.g., HSV-1 glycoprotein D, KRISHNA et al. 1989), while in other cases they may be less effective (e.g., bovine herpesvirus 1 glycoprotein gIV, VAN DRUNEN LITTEL-VAN DEN HURK et al. 1991). The effectiveness of insect cell-produced proteins as therapeutic agents remains a topic of debate, and can only be resolved through further experimentation.

4.3 O-Linked Glycosylation

There is less evidence in the published literature for O-linked glycosylation in this expression system. However, analysis of baculovirus-expressed pseudorabies glycoprotein gp50 in infected Sf-9 cells demonstrated the presence of O-linked polysaccharides by digestions with endo-alpha-*N*-acetylgalactosaminidase (THOMSEN et al. 1990). Subsequent analysis of the released disaccharide with bovine testis beta-galactosidase and jack bean beta-galactosidase suggested a beta 1–3 linkage between the monosaccharides. Enzyme assays confirmed the presence of N-acetylgalactosaminyl transferase and beta-1,3-galactosyl transferase activities in Sf9 cells (THOMSEN et al. 1990). Similar analyses were performed by WATHEN et al. (1991) in examining the oligosaccharide structures on a respiratory syncytial virus chimeric FG protein.

4.4 Fatty Acid Acylation

Both palmitoylation and myristoylation have been detected in recombinant-infected insect cells. Palmitoylation has been verified through labeling of a baculovirus-expressed SV40 large T antigen (LANFORD 1988), Ha-*ras* p21 (PAGE et al. 1989), and human transferrin receptor (DOMINGO and TROWBRIDGE 1988) with ^{3}[H]-palmitic acid. Similarly, myristoylation of the baculovirus-expressed preS-S polypeptide of the hepatitis B surface antigen (HBsAg; PERSING et al. 1987; LANFORD et al. 1988), the p17 *gag*-related protein of HIV (OVERTON et al. 1989), and the FIV *gag* precursor protein (MORIKAWA et al. 1991) was verified through labeling with ^{3}[H]-myristic acid. These reports confirm that the necessary enzymes for fatty acid acylation are present in baculovirus infected insect cells.

4.5 Nuclear Transport

A number of nuclear localized proteins have been expressed in the baculovirus system. One example is the human c-*myc* protein (MIYAMOTO et al. 1985). Baculovirus-expressed c-*myc* was phosphorylated, efficiently transported to the nucleus, and was found tightly associated with the nuclear matrix. These properties are identical to those of the native protein. Other phosphorylated

nuclear proteins that have been expressed are the *Kruppel* gene product of *Drosophila* (OLLO and MANIATIS 1987), the presumptive transposase of the Maize Ac transposon (HAUSER et al. 1988), and the mouse c-*ets*-1 protooncogene (CHEN 1990). Additional nuclear proteins successfully expressed include the human Ku autoantigen (ALLAWAY et al. 1990), human *fos* protein (TRATNER et al. 1990), and the *Egr*-1 transcription factor (RAGONA et al. 1991).

The expression of the qa-1F activator protein of *Neurospora crassa* demonstrated the effectiveness of employing a baculovirus late gene promoter for producing potentially cytotoxic gene products. The qa-1 activator protein was apparently toxic when expressed in either *E. coli* or *Saccharomyces cerevisiae*, but could be expressed when introduced into a baculovirus vector (BAUM et al. 1987). The recombinant protein had the same DNA-binding specificity as the native form, and was useful in determining the location of binding sites within the qa gene cluster.

4.6 Expression of Viral Nonstructural Gene Products

A number of viral nuclear proteins have also been successfully expressed. Recombinant polyomavirus large T antigen was antigenically similar to the mammalian form and displayed polyomavirus origin-specific DNA binding (RICE et al. 1987). The SV40 large T antigen was effectively phosphorylated and displayed origin-specific DNA binding as well (MURPHY et al. 1988). In addition, the SV40 large T antigen displayed an ATPase activity comparable to that of the mammalian cell-derived counterpart, indicating retention of significant elements of the native protein conformational structure (MURPHY et al. 1988). A similar analysis of SV40 T-antigen expression by LANFORD (1988) detected palmitylation, glycosylation, and oligomerization of T-antigen produced in baculovirus-infected insect cells. All of these modifications of recombinant large T antigen are found in the native protein isolated from mammalian cells (KLOCKMANN and DEPPERT 1983).

Transactivating gene products from a number of mammalian viruses have been expressed and characterized using the baculovirus system. These include the herpes simplex virus (HSV) alpha-TIF regulatory protein (KRISTIE et al. 1989) and trans-activator Vmw65 (CAPONE and WERSTUCK 1990), the human T-cell leukemia virus type I (HTLV-I) $p40^{x}$ trans-activator (NYUNOYA et al. 1988; JEANG et al. 1987b), the bovine leukemia virus (BLV) $p34^{tax}$, and the hepatitis B virus X protein (SPANDAU et al. 1991).

The transactivating $p40^{x}$ protein of HTLV-I is phosphorylated (NYUNOYA et al. 1988; JEANG et al. 1987b) and can transactivate an HTLV-I LTR indicator target plasmid in recombinant-infected insect cells (JEANG et al. 1987b). In contrast, the recombinant transactivating protein, *tat*, of the human immunodeficiency virus (HIV) type 1 was not phosphorylated and its biological activity could not be assessed directly in infected Sf-9 cells (JEANG et al. 1988a). Instead, Sf-9 cells infected with the *tat* recombinant virus were fused with a mammalian

cell line containing an integrated HIV LTR-CAT reporter plasmid. The detection of CAT activity following fusion of the baculovirus-infected insect cells and the transformed mammalian cells confirmed the biological activity of the recombinant *tat* protein (JEANG et al. 1988a). Activity of recombinant HTLV-I and HIV-1 *tat* proteins was evident in the absence of de novo cellular protein synthesis (JEANG et al. 1988b). These studies established cell fusion as an effective strategy for assessing the biological acitivity of recombinant proteins synthesized in insect cells in a mammalian cell environment.

The baculovirus-infected insect cell can provide large quantities of easily purified mammalian virus proteins for research purposes. Recombinant dengue virus 4 structural and nonstructural proteins may be useful in defining epitopes that are protective from those that are immunopathogenic (ROTHMAN et al. 1989). Recombinant baculoviruses have been utilized extensively to facilitate genetic and biochemical analyses of the function and activities of animal virus gene products. These include the adenovirus type 2 DNA polymerase (WATSON and HAY 1990), polyoma virus middle T antigen (FORSTOVA et al. 1989), poliovirus $3B^{VPg}$, $3C^{pro}$, and $3D^{pol}$ (NEUFELD et al. 1991), the human papillomavirus type 18 E6 protein (GROSSMAN et al. 1989), the herpes simplex virus UL5, UL8, UL9, and UL52 gene products (OLIVO et al. 1988, 1989) and DNA polymerase (MOARCY et al. 1990), and several different HIV-1 *pol* gene products (HU and KANG 1991). While the baculovirus-produced Epstein-Barr virus alkaline deoxyribonuclease will be useful for biochemical analyses, it may also be an effective reagent for diagnosis of nasopharyngeal carcinoma (BAYLIS et al. 1991).

4.7 Expression of Virus Structural Proteins

Numerous mammalian virus capsid protein genes have been expressed for potential use as diagnostic or vaccine reagents. A cDNA encoding the major rotavirus capsid protein VP6 was efficiently expressed and formed trimeric capsid subunits and oligomeric assemblies (ESTES et al. 1987). All of the native immunoreactive determinants were conserved as assessed by reactivity with a battery of monoclonal antibodies. In addition, antiserum prepared against baculovirus-produced VP6 was highly specific for the viral protein, and lacked reactivity with mammalian proteins (ESTES et al. 1987). These results suggested potential advantages for use of baculovirus-produced proteins in diagnostic test kits.

The expression of other rotavirus gene products has facilitated characterization of some of their properties or functions. Examples include the outer capsid protein VP4 of the rhesus rotavirus which was characterized as the virus-neutralizing antigen and hemagglutinin (MACKOW et al. 1989), the bovine rotavirus VP1 (COHEN et al. 1989), and the Simian rotavirus nonstructural phosphoprotein product of gene 11 (WELCH et al. 1989). Passive immunization of suckling mice could be effected by inoculating female mice with baculovirus-expressed VP4 (MACKOW et al. 1990).

Several bluetongue virus (BTV) gene products have been analyzed following expression (INUMARU and ROY 1987; INUMARU et al. 1987; URAKAWA and ROY 1988; FRENCH et al. 1989; OLDFIELD et al. 1990; THOMAS et al. 1990). The recombinant VP2 protein of BTV serotype 10 was capable of inducing neutralizing antibody titers in both mice and rabbits, and might be useful as a subunit vaccine (INUMARU and ROY 1987), while recombinant VP5 was incapable of eliciting a neutralizing antibody response (MARSHALL and ROY 1990). The recombinant BTV VP3 and VP7 group-specific antigens are effective in detecting several BTV serotypes by indirect ELISA and may be useful as diagnostic reagents (INUMARU et al. 1987; OLDFIELD et al. 1990). Baculovirus expression of NS1, another group-specific antigen, demonstrated that this protein forms the virus-specific tubules seen during BTV-10 infections (URAKAWA and ROY 1988; MARSHALL et al. 1990), while the NS2 gene product is the structural component of the virus inclusion bodies characteristic of BTV-10 infections (THOMAS et al. 1990). Both of these structures are formed in the recombinant baculovirus-infected insect cells as well.

Several other recombinant baculovirus-produced viral proteins show promise of being effective diagnostic antigens. Examples include Hantaan virus structural proteins (SCHMALJOHN et al. 1988), hepatitis virus core and surface antigens (TAKEHARA et al. 1988), the hepatitis A VP1 capsid protein (HARMON et al. 1988), a dengue 4 virus core/PreM fusion protein (MAKINO et al. 1989), the Lassa fever virus nucleocapsid protein (BARBER et al. 1990), the hepatitis C virus core protein (CHIBA et al. 1991), human papillomavirus (HPV) 6b E2 gene product (SEKINE et al. 1989), the L2 open reading frames of both HVP 6b and 11 (ROSE et al. 1990), the human parvovirus B19 VP1 and VP2 structural proteins (BROWN et al. 1990), rabies virus nucleoprotein (PREHAUD et al. 1990), and the gp57-65 of Marek's disease virus (NIIKURA et al. 1991b), which may also be useful as a vaccine reagent.

With respect to producing antigens for diagnostic assays, ROTA et al. (1990) point out that one advantage of producing influenza A and B nucleoprotein antigens in recombinant baculovirus-infected insect cells is that the proteins need not be purified prior to use in ELISA assays. They were unable to detect antibodies in human, mouse, ferret, rabbit, or chimpanzee sera that reacted with control baculovirus-infected insect cells. MILLS and JONES (1990) produced the p24 core protein of HIV and were able to enrich the recombinant protein to greater than 90% purity from soluble fractions of infected insect cells in a simple two-step procedure of ammonium sulfate precipitation and gel filtration. The resulting preparation was capable of detecting p24 antibodies in all AIDS patients' sera tested and exhibited no background reactivity with serum from a noninfected individual. Similar low levels of background reactivity were reported by DEVASH et al. (1990) in Western blots to detect antibodies towards baculovirus-produced HIV *rev* and *vif* gene products in ARC and AIDS patients' sera.

The HIV *gag* region and a *gag*/*pol* segment including the entire *gag* region and 65% of the *pol* gene were each expressed in this system (MADISEN et al.

1987). The results indicate that initial proteolytic processing of *gag* from the 55-kDa precursor polypeptide to the 40-kDa precursor is apparently mediated by cellular proteases common to both insect and mammalian cells. Subsequent proteolysis of the p40 polypeptide to mature viral proteins p24, p18, and p14 is mediated by a *gag*-specific protease activity encoded by the *pol* region. The recombinant *gag* polypeptides were specifically recognized by AIDS patient serum suggesting they could serve as useful diagnostic reagents (MADISEN et al. 1987).

The $Pr55^{gag}$ precursor protein of HIV-1 assembles into virus-like particles (VLPs) in recombinant baculovirus-infected insect cells (GHEYSEN et al. 1989). The particles are targeted to the cell membrane and mutagenic analyses established that myristoylation of the amino-terminal glycine residue is essential for budding of spherical particles from the cell. Similar analyses were performed on simian immunodeficiency virus $Pr57^{gag}$ (DELCHAMBRE et al. 1989).

VLPs that were myristoylated and secreted were also obtained upon expression of the feline immunodeficiency virus (FIV) *gag* precursor protein. Inclusion of the FIV protease gene with the *gag* precursor gene allowed processing of the *gag* precursor and no VLPs were released (MORIKAWA et al. 1991). VLPs formed during expression of the bovine immunodeficiency virus precursor, $Pr53^{gag}$, could be processed in vitro utilizing the protease activity from NP-40-lysed preparations of purified BIV virions (RASMUSSEN et al. 1990). These studies point to the utility of these baculovirus-produced precursor *gag* VLPs for structural studies and for analysis of inhibitors of viral-specific protease activity, a necessary function in the maturation of infectious virions.

The ability to produce noninfectious VLPs using baculovirus expression vectors offers the potential for generating safe vaccine preparations. Noninfectious empty capsids are produced during coexpression of human parvovirus B19 structural proteins VP1 and VP2, or upon expression of VP2 alone (BROWN et al. 1991; KAJIGAYA et al. 1991). However, only particles containing VP1 were capable of inducing a neutralizing antibody response immunized rabbits (KAJIGAYA et al. 1991). Correct processing of capsid precursor polyprotein P1 into VP0, VP1, and VP3 occurred during expression of the complete coding region of poliovirus type 3. The capsid proteins assembled into noninfectious VLPs that were able to induce neutralizing antibodies in immunized mice (URAKAWA et al. 1989). Expression of the foot-and-mouth disease virus P1-2A region in the presence of the 3C protease yielded correct cleavage of the P1-2A polyprotein into capsid proteins 1AB, 1C and 1D but the efficiency of aggregation into particles was low (ROOSIEN et al. 1990).

Cowpea mosaic virus genes were also successfully expressed in baculovirus-infected cells, and at higher levels than that obtained for the same genes in bacteria. Correct proteolytic processing of precursor polypeptides by the expressed recombinant 24 K viral protease also occurred (VAN BOKHOVEN et al. 1990).

4.8 Other Proteins of Interest

The uniqueness of the baculovirus-infected insect cell environment, coupled with the characteristics of high-level expression, have induced many investigators to use the system for biochemical investigations of protein structure and function. Tyrosine hydroxylase, implicated in the pathogenesis of neuropsychiatric disorders, has been expressed for analyses of its biochemical and pharmacological properties (GINNS et al. 1988; FITZPATRICK et al. 1990). Human 5-lipoxygenase (FUNK et al. 1989) and human terminal transferase (CHANG et al. 1988) were expressed at relatively high levels and retained their enzymatic activities in insect cells. The function and structure of various domains of the insulin receptor have also been examined (ELLIS et al. 1988; HERRERA et al. 1988; VILLALBA et al. 1989; PAUL et al. 1990).

A variety of protein kinase genes or kinase domains of membrane receptors have been shown to retain their respective activity and specificity (WEDEGAERTNER and GILL 1989; PATEL and STABEL 1989; BRICKEY et al. 1990). Several G protein-coupled receptors were localized in the insect cell membrane and retained their activity and selectivity (PARKER et al. 1991; REILANDER et al. 1991).

A few insect-related proteins have been expressed, including the firefly luciferase gene (HASNAIN and NAKHAI 1990) and attacin (GUNNE et al. 1990), and two plant proteins, patatin and beta-phaseolin, have also been efficiently expressed (ANDREWS et al. 1988; BUSTOS et al. 1988).

The general application of gene transfer and expression in baculoviruses has renewed interest in their potential as microbial insecticides. Several different approaches involving expression of insect-specific toxins have been examined by various investigators. CARBONELL et al. (1988) describe an unsuccessful attempt to use the insect-specific scorpion toxin. A similar approach using the insect-specific scorpion neurotoxin gene of a different species proved more successful (STEWART et al. 1991) and reduced the time required for the virus to kill the insect host.

MAEDA (1989b) describes the use of a diuretic hormone from *Manduca sexta* to engineer recombinant BmMNPV which kill infected larvae 20% faster than the wild-type virus in injection experiments. Introduction of a recombinant baculovirus expressing the juvenile hormone esterase (JHE) gene from *Heliothis virescens* proved somewhat effective in reducing feeding behavior of first-instar *T. ni* larvae, but due to the instability of the JHE in vivo, was less effective on later stages (HAMMOCK et al. 1990).

MARTENS et al. (1990) engineered AcNPV to express the *Bacillus thuringiensis* crystal endotoxin gene *cryIA*(b) in place of the polyhedrin gene. The CryIA(b) protein produced in baculovirus-infected *S. frugiperda* cells formed crystals that were toxic to a susceptible insect species.

A newly isolated gene encoding a mite neurotoxin shows promise of being useful for enhancing efficacy of recombinant baculoviruses for control of insect

pests. Recombinant baculoviruses producing the toxin were effective in paralyzing fifth-instar larvae within 2 days of injection, while larvae infected with the same dose of wild-type virus continued feeding (TOMALSKI and MILLER 1991). As other insect-specific toxins are identified, the prospects for utilizing recombinant baculoviruses as pest control agents will continue to improve.

5 Scale-Up Considerations

The lepidopteran cell lines commonly employed for growth and expression of baculovirus vectors are relatively hardy. Cultures can be maintained at room temperature if desired, although the optimal growth temperature is 27° to 29° C. Insect cell culture media are not buffered with carbonate/CO_2 so there is no requirement for a CO_2 environment for growth. A variety of media have been formulated for insect cell growth. The most commonly employed are Grace's antherea medium (GRACE 1962), Hink's TNM-FH modification of Grace's medium (HINK 1979), TC100 (GARDINER and STOCKDALE 1975), and IPL41 (WEISS et al. 1981), all of which require some serum supplementation. Several companies have developed effective serum-free media for insect cells (e.g., EX-CELL 400, JRH Biosciences, Lexana, Kan.; SF900, GIBCO/BRL, Grand Island, N.Y.) that are rapidly replacing the serum-supplemented formulations. However, these serum-free formulations may not support the growth of all lepidopteran cell lines.

HINK et al. (1991) examined the relative levels of expression of three proteins, beta-galactosidase, human plasminogen, and respiratory syncytial virus gp50T, in 23 lepidopteran cell lines each cultured according to original descriptions. The analysis demonstrated that no individual cell line could be expected to produce optimal levels of all three proteins, and optimal expression of individual proteins for production purposes may require examination of several lepidopteran cell lines.

The expression of recombinant proteins by baculovirus-infected insect cells is efficient enough that 1- to 5-l batch cultures usually suffice for most laboratory scale-up purposes. Roller bottles, air-sparged suspension cultures, and air-lift bioreactors all have proven successful for these purposes (HINK 1982; WEISS et al. 1988). The most convenient method for preparing 1- to 5-l batch cultures is to utilize spinner or stirrer culture setups (HINK 1982; WEISS et al. 1988). These types of cultures are capable of providing cell densities of up to 4 or 5×10^6 cells/ml. Optimal expression of foreign gene products is attained by inoculating during the log phase of the spinner culture growth at densities of around 1×10^6 cells/ml.

The important parameters for suspension cultures of insect cells are shear stress, dissolved oxygen content, and pH (HINK 1982). In a 1-l (or less) batch culture, the pH need not be monitored, assuming that cells are added to fresh

medium upon initiating the culture. However, the addition of oxygen to the culture medium by gentle aeration is required to insure optimal cell growth and virus replication (HINK 1982).

The virus inoculum may be added at multiplicities of 0.1 or 1 plaque-forming units (pfu) per cell to log phase cultures of 1×10^6 cells/ml. At these multiplicities the culture may go through one more doubling before all cells become infected. As a conservative estimate, a 1-l culture can be expected to generate 1–10 mg expressed protein product, although levels as high as 200 mg/l are reported (JEANG et al. 1987b).

The product may be harvested between 48 and 72 h p.i., with some attention given to the rate of degradation of product during the course of the infection. LICARI and BAILEY (1991) suggest that as the infection of cells in a bioreactor progresses less cellular proteolytic activity may be evident and the amount of degraded protein product may actually decrease.

Larger scale cultures for the production of baculovirus-expressed proteins are feasible. Air-lift fermenters of up to 30 or 40 l can be employed with either serum-containing or serum-free media to effect large-scale propagation of cells and production of the baculovirus-expressed gene products (MAIORELLA et al. 1988). The first demonstrated serum-free medium, ISFM (INLOW et al. 1989), was based upon the IPL-41 formulation and was effective for both small-scale (less than 10 l) and large-scale (greater than 20 l) production of human macrophage colony stimulating factor (M-CSF) in baculovirus-infected Sf-9 cells (MAIORELLA et al. 1988). The medium substituted a lipid emulsion containing cod liver oil, cholesterol, alpha-tocopherol acetate, and Tween 80 (Sigma) for the serum component, and incorporated pluronic polyol F-68 (BASF Wyandotte) to reduce shear stress on the cells. A 10000-MW filtrate of TC yeastolate reduces additional high-molecular-weight proteins in the medium. Pluronic F-68 has been demonstrated effective in reducing shear dammage to cultured insect cells in agitated, sparged, and air-lift bioreactors (MURHAMMER and GOOCHEE 1988) and is now considered a routine additive for large-scale cultures.

The ISFM formulation provided similar levels of cell growth and recombinant protein production to the serum supplemented IPL-41 medium. Up to 40 mg/l of recombinant M-CSF was produced following infection at cell densities of 3×10^6 cells/ml with a multiplicity of 1 pfu/cell (MAIORELLA et al. 1988). Additional recombinant proteins produced in this manner included plasminogen activator and ricin toxin (see MAIORELLA et al. 1988). These results established that large-scale serum-free cultivation of insect cells for the production of baculovirus-expressed proteins is entirely feasible and can be cost effective. Tissue culture-tested components for the ISFM media formulation are also commercially available (Sigma Chem. Co., St. Louis, Mo.).

Because the virus infection ultimately causes cell death, production cultures are necessarily batch-type rather than continuous. Sequential batch situations for semicontinuous cycling of cells can be envisioned (HINK 1982), but cycling of virus inoculum is not advised due to the problems associated with continued serial propagation of the virus and the generation and amplification of mutations

(BURAND and SUMMERS 1982; KUMAR and MILLER 1987). Each batch culture should be inoculated with virus preparations derived from a stock inoculum that originated from a well-characterized plaque-purified virus. The virus may be stored as infected cell culture supernatants at 4° C for prolonged periods (several months to a year or more) without significant loss of activity. Cell cultures should be started from frozen stocks and used within a defined number of passages. These strategies maximize consistency, and ultimately productivity, for the batch scale-up process.

Finally, several investigators have suggested the possible use of insect larvae as bioreactors for large-scale production of baculovirus-expressed gene products. The silkworm, *B. mori*, has proven an effective alternative to the BM-N cell line (MAEDA et al. 1985; HORIUCHI et al. 1987; MARUMOTO et al. 1987; MIYAJIMA et al. 1987; MAEDA 1989a; TADA et al. 1988; MORISHITA et al. 1991) for production of baculovirus-expressed proteins. The silkworm host is an example of a completely domesticated animal, incapable of survival outside of the laboratory, and thus provides a biological level of containment. The larvae grow to be quite large, and rearing on semisynthetic diets can be automated (MAEDA 1987). As much as 0.3 ml of hemolymph (blood) can be harvested from a single larva, with levels of 30–190 μg protein product/ml (MAEDA et al. 1885; HORIUCHI et al. 1987). The stability of crude preparations of hemolymph appears to be quite good at 4° C, as IL-3 exhibited no loss in activity after storage for several days (MIYAJIMA et al. 1987). Whether all proteins will be as stable remains to be determined.

Strategies have also been developed for production of proteins in *T. ni* larvae (KURODA et al. 1989; PRICE et al. 1989; MEDIN et al. 1990). PRICE et al. (1989) demonstrated the effectiveness of coinfection with wild-type and recombinant baculoviruses to generate infectious OBs containing virions of both viruses, thus increasing the effectiveness of inoculating insect larvae by feeding.

Purification of product from larval hemolymph apparently presents no unusual problems. Both alpha-interferon and IL-3 could be effectively purified by affinity chromatography. In fact, the only difficulty appears to be associated with harvesting by bleeding individual larvae. Presumably an alternative method would be employed in production level scale-up.

6 Concluding Remarks

The use of baculoviruses for expression of eurkaryotic gene products has gained wide acceptance as an attractive alternative to other eurkaryotic expression systems. This is largely due to the ease of handling of the virus and its host cell cultures, and the relatively high levels of expression that can be effected in laboratory-scale production. In addition, the overwhelming majority of recombinant proteins produced by the baculovirus expression system retain the

antigenic and biological properties of the native proteins. At the very least, the baculovirus system will continue to be a useful addition to the expression vector repertoire.

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