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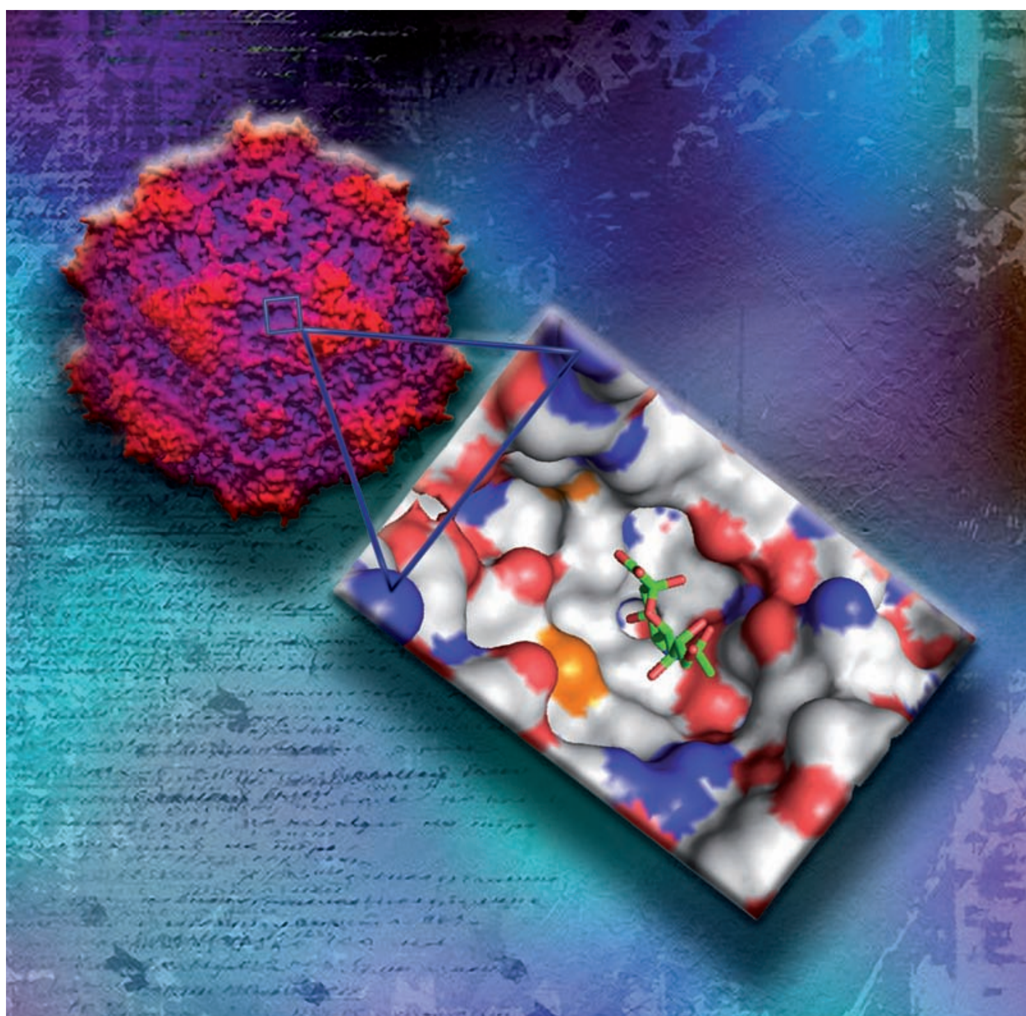
Structural Virology



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Structural Virology

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Preface

Viruses can be grouped among the simplest biological systems that have the ability to evolve and adapt to exist in different environments. That is, they have the ability to 'jump' from one host to another, some carrying the necessary molecular machinery to transfer and modify their genetic information from one generation to the next, while others hijack the host machinery to effect the necessary modifications. Because of this innate ability, it would not be unreasonable to state that viruses have most likely infected every life form that has ever existed on our planet, from the simplest single-cell organisms to plants, animals, and humans.

To achieve such biodiversity, viruses have evolved different and efficient strategies for host recognition, internalization, cellular trafficking, genome replication, capsid assembly, genome packaging, release of progeny (for re-infection) and host immune surveillance evasion, to optimize their life cycle in their unique niche. This has resulted in viruses of different shapes and sizes, from simple single-protein spherical or helical assemblages, to multiple complex systems, assembled from hundreds of proteins without/with (enveloped) the incorporation of host lipids. Invariably the viral coat protein(s) (referred to throughout this monograph interchangeably as either CPs or VPs) form some sort of integral protective shell (a viral capsid) around the infectious genomic nucleic acid, which can be single-stranded (ss) DNA, ssRNA, double-stranded (ds) DNA or dsRNA, packaged as single or multiple, linear or circular molecule(s). The packaged viral genome encodes all the required structural CPs/VPs and auxiliary non-structural proteins that are required in combination with host proteins for host infection. The enveloped viruses incorporate their host's lipids as either an internal and/or external envelope during their assembly. For a number of viruses, CP/VP recognition and encapsidation of the genomic nucleic acid is a prerequisite for infectious capsid formation, whereas for others the genome is packaged into preformed capsids via interactions with viral or host encoded proteins. In addition to genome encapsidation and protection during cellular entry and trafficking, the CP/VP can also dictate many other

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viral functions, including host receptor/vector recognition, transmission and the genomic transduction efficiency during infection.

For spherical viruses, the CP/VP organization in the capsid architecture takes on the form of an icosahedron (a platonic solid with point group symmetry 5.3.2), a regular polyhedron which is assembled from 20 equilateral triangles. This symmetrical shell is a consequence of it consisting of identical (or almost identical) gene products, consistent with the argument that there is insufficient volume inside a virus to accommodate a more complicated protein coding strategy. The exact twofold, threefold and fivefold symmetry of the icosahedron permits the (quasi) equivalent symmetry required to construct structures with 60 or multiples [denoted by a T (triangulation) number] of 60 subunits. This monograph will discuss viruses assembled from the simplest of icosahedral capsids, with $T = 1$ triangulation (assembled from 60 CP/VP subunits), to those with more complicated VP shells assembles and lipid membrane envelopes.

Viruses have been responsible for more human deaths, either through direct infection (such as influenza virus) or infection of crops, than any other known human disease-causing agent. In addition, their ability to package efficiently and deliver genomic material to different living organisms and tissues also makes them attractive vehicles for the delivery of therapeutic genetic material in situations where defective genes lead to disease phenotypes. Thus viruses are the subject of intense scientific study in many different disciplines, including structure biology, in efforts to (i) understand the basic biological processes governing viral infection and (ii) develop treatment strategies, including vaccines, anti-virals and gene delivery vectors.

The use of structure approaches in virology has given insight into the structural basis of assembly, nucleic acid packaging, particle dynamics and interactions with cellular molecules and allowed the elucidation of mechanistic pathways at the atomic and molecular level. Biological processes, such as the life cycle of a virus infection, are governed by numerous intricate macromolecular interactions. The role of the structural virologist is thus to visualize these interactions in three dimensions (3D), to provide a full understanding of these interactions as 'seeing is believing'. These structural characterizations of viruses then provide crucial platforms for the development of treatment and therapeutic strategies (Section 3 of this monograph).

The range of biophysical methods used in structural virology is vast, ranging from hydrodynamic to scattering techniques (Section 1 of this monograph), and have played a fundamental role in our understanding of viral infection in recent years. The method undertaken for a particular study is often dependent on the resolution and type of information desired and also the size and complexity of the macromolecule under investigation, the amount of material available, its solubility in aqueous environments (Chapter 1) and the type of interactions being visualized. For example, for the imaging of whole viruses during infection, confocal microscopy (Chapter 2) and cryo-electron tomography (cryo-ET) (Chapter 4) are applied, which permit studies at molecular resolution. And while both nuclear magnetic resonance (NMR) spectroscopy (Chapter 8) and X-ray crystallography (Chapters 6 and 7) can give atomic resolution detail on protein

backbone and side-chain placement, NMR also provides dynamic (ensemble) information and crystallography provides a 'snapshot' and is often considered static. Solution approaches, such as limited proteolysis combined with mass spectrometry and small-angle scattering approaches (Chapter 3), also provide dynamic information. In cryo-ET and cryo-electron microscopy (cryo-EM) (Chapter 5), macromolecules are frozen in their native state, allowing for discrete selection of dynamic states to be visualized, albeit at lower resolution. Generally, NMR spectroscopy is utilized for small protein molecules that are flexible, X-ray crystallography for medium-sized proteins and complexes that are compact, whereas very large macromolecular assemblages or membranous protein structures are determined by cryo-EM. The largest issue separating cryo-EM and cryo-ET from crystallography, in addition to size and the limitations of crystal formation, is resolution. Cryo-EM has generally been considered a low-resolution technique, giving reconstructions around 15–30 Å, but with advances in sample handling, instrumentation, image processing and model building, near-atomic resolution structures are now being achieved. For cryo-ET the resolution achieveable is still low.

In reality, hybrid approaches, combining NMR, X-ray crystallography and cryo-EM, cryo-ET and solution data, are often adopted, which provides a powerful means of filling gaps which can arise in the structural characterization of large macromolecules. For example, in studies where large viruses cannot be crystallized, subcomponents can be crystallized to obtain high-resolution information, which can then be used to interpret the structure at lower resolution obtained by cryo-EM or cryo-ET. Or atomic structures obtained from homologous viral proteins/virus capsids can be used for 3D homology model building. These approaches permit the pseudo-atomic visualization of interaction interfaces between protein–protein subunits, protein–nucleic acids and protein–lipid in virus capsids and also the visualization of virus capsid–host interactions.

Combined with biochemical, biophysical and molecular biology analysis, structural studies indicate a high degree of fidelity in the steps that result in the assembly of mature infectious virus capsids (Chapter 10). They also show that the fundmental principles governing successful viral capsid assembly, efficient polymerization of CP subunits utilizing specific interface interactions that spontaneously terminate, often employ structural polymorphisms to facilitate the required interactions. Structural virology approaches have also been platforms for the elegant description of the virus infection process, from initial receptor attachment to the interaction of the capsid with host antibodies (Section 2), and provided the targets for therapeutic intervention and improved viral capsid vectors for gene delivery (Section 3).

This monograph is designed to provide a basic introduction to the use of structural virology and its applications in virus research towards functional annotation and is not intended to provide a detailed discussion of approaches utilized.

Mavis Agbandje-McKenna
Robert McKenna

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Section 1

CHAPTER 1

Production and Purification of Viruses for Structural Studies

BRITTNEY L. GURDA AND MAVIS AGBANDJE-MCKENNA

Department of Biochemistry and Molecular Biology, Center for Structural Biology, The McKnight Brain Institute, University of Florida, Gainesville, FL 32610, USA

1 Introduction

Advances in protein production and purification techniques over the past two decades have allowed the structural study of numerous proteins and macromolecular assemblages that would have otherwise been intractable to the necessary approaches (detailed in the following chapters). This chapter focuses on the production and purification of intact viral capsids (particles) with/without genome for structure determination. The production and purification of viral proteins for structure determination by X-ray crystallography and NMR spectroscopy are the subjects of Chapters 7 and 8, respectively. Crystallization is often considered a method of purification and a function of purity, often of a protein or virus capsid, and, as such, sample preparation for structure determination by X-ray crystallography places high demands on sample quality. Screening trials to identify the optimal crystallization conditions also require large quantities of sample compared with the majority of other structure determination approaches discussed in the subsequent chapters of this monograph. Virus samples produced for such analyses also have to be both stable and soluble in their storage buffer since degradation and aggregation are detrimental to the crystallization process. Hence this chapter will focus

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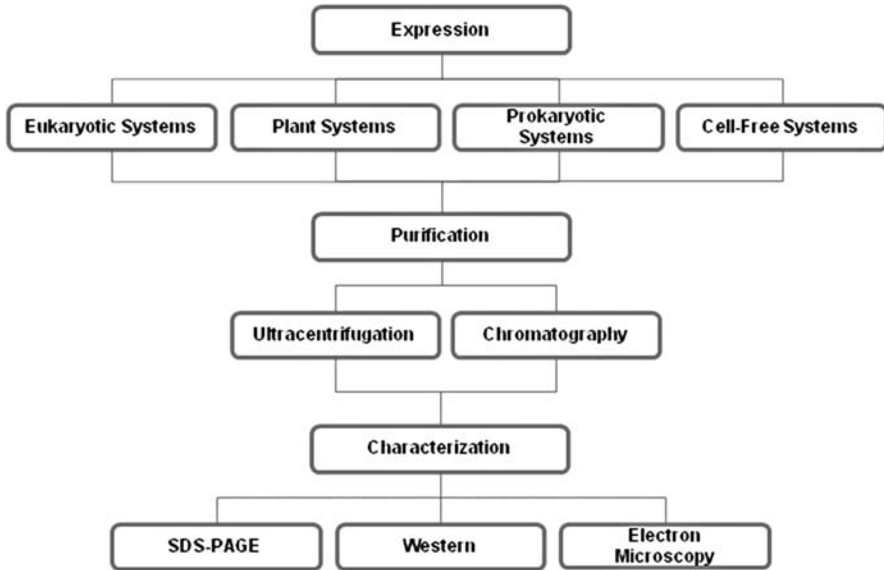

Figure 1 The steps involved in the expression, purification and characterization of virus capsids prior to structural analysis.

on methodologies to produce and purify virus capsids (Figure 1) in quantities suitable for structure determination by X-ray crystallography, with the premise that such a sample would also be suitable for structural or biophysical analysis using other methodologies.

2 Expression Systems

Most viruses are considered hazardous material in their wild-type (wt) infectious form (for information on safe handling and containment of infectious microorganisms and hazardous biological materials, see http://www.cdc.gov/biosafety) and are therefore often studied in a recombinant form. Significant effort has been extended into the development of heterologous expression systems to produce recombinant viral proteins which will assemble into viral capsids. The system selected for use is often dependent on the properties of the viral genes and the environmental requirements of the final product. However, the most important factor to consider is the capacity of the host cells to translate the RNA transcript, to ensure proper folding of the gene product and to sustain the protein(s) expressed in an intact and functional state.[1] Protein expression systems contain at least four general components: (1) the genetic elements necessary for transcription/translation and selection; (2) in vector-based systems, a suitable replicon: plasmid, virus genes, *etc.*; (3) a host strain containing the appropriate genetic traits needed to function with the specific

expression signals and selection scheme; and (4) the culturing conditions for the transformed cells or organisms.[2]

Eukaryotic Systems

Mammalian Cells

Since most viruses currently studied are of human or animal origin, mammalian tissue culture is an ideal source to generate viral capsids for structural studies which are generally aimed at functional annotation. In this system, proper folding is achieved and modifications such as complex glycosylation, phosphorylation, acylation, acetylation and γ-carboxylation are obtained. However, yields can be low, depending on gene product(s), ranging from 0.1 to $100\,\mathrm{mg\,L^{-1}}$ of culture volume. For some of the structural approaches discussed in Section 1 of this monograph, low yields may not be a problem since small amounts of sample are adequate. However, low yields can become problematic in crystallization, especially with a virus that does not have an established crystallization condition. In such a situation, numerous preparation steps may be required to obtain the quantities needed to screen crystallization conditions efficiently. Supplies and reagents can then become expensive, depending on individual cell line requirements. In addition, considerable time and resources can be spent on the construction of a suitable expression system and equally on optimization for suitable yields. In such situations, it is always advisable to seek the expertise of an established molecular biologist before designing new constructs.

Established cell lines and protocols exists for many different tissue systems and, although most of these cell lines are derived from human or mouse tissues, other mammalian cell culture lines are available, such as monkey, raccoon, horse, pig and rabbit. The American Type Culture Collection (ATCC) has over 3400 cell lines from 80 different species, including over 950 cancer cell lines (http://www.atcc.org/). Other cell suppliers include the Health Protection Agency Culture Collections (HPACC; http://www.hpacultures.org.uk/), the German Research Center for Biological Material (DSMZ; http://www.dsmz.de/) and the Riken BioResource Center Cell Bank (Riken; http://www.brc.riken.jp). It is strongly recommended that investigators purchase cell lines from recognized centers such as these listed above to ensure pure, authentic and quality controlled cell lines. The decision to use cells directly from an organism, *i.e.* primary cells or an immortalized cell line, should be based upon requirements of the virus system and available current protocols. As discussed below, there are three main approaches for virus production in mammalian cell lines: (i) infection of permissive cell lines with wt virus, (ii) transfection of cells with plasmid constructs containing viral genome sequences and (iii) viral vector systems which expression heterologous viral genes.

Although the majority of viruses currently studied are obtained from recombinant expression systems (see below), direct infection of cell lines with wt virus can be used to generate suitable quantities of sample for structural studies under certain conditions and for well-characterized viral systems.

For example, the human rhinovirus 3 (HRV3) virion particles used for determining its structure were purified from virus-infected HeLa cells (immortalized human cancer cells). The atomic structure of HRV3 was initially determined to 3 Å,[3] and later refined to 2.15 Å.[4] It was reported that 10–12 L of HeLa cells (at 6–8 $\times 10^5$ cells mL^{-1}) were used to generate the amount of virus necessary to carry out crystallization and structure determination. Echovirus-1, also of the *Picornaviridae* family, was also successfully produced in HeLa cells for its structure determination to $\sim$3.55 Å resolution.[5]

In the use of plasmid constructs, one or more plasmids usually containing capsid proteins alone and, if needed, replication factors, are used to transfect cells, which results in the assembly of virus-like particles (VLPs). Often, another plasmid is added when a packaged gene is desired, *e.g.* reporter gene, or if genome is needed to produce stable virions. Recovered virus can either be purified for structural studies or, if infectious, used to infect permissive cells for continual propagation of virions. As an example, molecular clones containing the capsid sequence of canine parvovirus was used for the transfection of Norden Laboratories feline kidney cells (NLFK)[6] to produce particles for X-ray crystallographic structural studies to 3.2 Å resolution.[7] For the crystallographic structure determination of the immunosuppressive strain of minute virus of mice (MVMi), infectious virions were harvested from plasmid transfected cell lines and subsequently propagated in a permissive cell line to produce virus for crystallization.[8]

The development of heterologous surrogate expression systems for virus capsid production has enabled researchers to overcome the lack of efficient expression in homologous systems for several viruses of interest. As an example, for hepatitis C virus (HCV), a herpes simplex virus-1 (HSV-1)-based amplicon vector system that expresses HCV capsid proteins and the two envelope proteins, E1 and E2, under the HSV-1 IE4 promoter was developed.[9] This system has several advantages; (i) the ability to infect a wide range of cells, without the limitation of transfection efficiency, including primary cells in a quiescent state, (ii) the simplicity of cloning desired genes into amplicons, (iii) the high capacity of incorporation of exogenous sequences in the vector genome and the transfer of high copy numbers of the exogenous gene and (iv) the potential for using amplicons in vaccine design and development.[10] A mini-review has covered HSV amplicons from genomes to engineering.[11] Norovirus is another example of a non-cultivable virus that remained refractory to structural studies due to the lack of a reverse genetics system and a permissive cell line until recent advances. A novel expression strategy, which combined the use of a two baculovirus transactivation system to deliver viral cDNA and an inducible DNA polymerase (pol) II promoter, led to the ability to grow this virus in several cell lines, including HepG2, BHK-21, COS-7 and HEK293T cells.[12,13]

Yeast Cells

Among the microbial eukaryotic host systems, yeasts can combine the advantages of unicellular organisms (*e.g.* ease of genetic manipulation and

growth) with the capabilities of a protein processing typical of eukaryotic organisms (*e.g.*, protein folding, assembly and posttranslational modifications).[14] The majority of recombinant proteins produced in yeast have been expressed using *Saccharomyces cerevisiae*. More commonly referred to as baker's or budding yeast, *S. cerevisiae* was the first eukaryote to have its entire genome sequenced[15] and is still today considered a model organism. A scientific database has been established for *S. cerevisiae* and is available at http://www.yeastgenome.org/. With its biochemistry, basic genetics and cellular biology already well established, this simple eukaryote has become a major tool in answering questions of fundamental biological importance and is a central player in post-genomics research.

Appealing aspects of the yeast expression system are its rapid cell growth (with a doubling time of ~90 min), simple growth media, secretion of recombinant proteins to the medium and glycosylation capability. N-linked glycosylation is minimal with high mannose, but O-linked modifications appear similar to mammalian cells. Phosphorylation, acetylation and acylation are also present. Protein yields are comparable with the baculovirus system (see below) at ~10–200 mg L^{-1} depending on recombinant gene properties. Issues in large-scale protein production involving *S. cerevisiae* appear to be hyperglycosylation and retention in the periplasmic space.[16,17] This ultimately leads to a loss of final protein due to retention and degradation. The search for alternative hosts has led to the use of 'non-conventional' yeasts in expression protocols. The most established examples include *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Pichia methanolica*, *Pichia stipitis*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces bailaii*, *Candida boidinii* and *Schwanniomyces* (*Debaryomyces*) *occidentalis*.[14] These systems are broken down even further into two categories: methyltrophic, *e.g. P. pastoris*, and non-methyltrophic, *e.g. S. cerevisiae*. These categories are based on the fermentation processes involved and generally dictate the promoter that should be used in the experimental design. The choice of yeast host is one of the most important determinants of the success of the entire project, and many reviews debating the subject can be found in the current literature. Generally, the expression of foreign proteins in yeasts consists of (i) cloning of a foreign protein-coding DNA sequence within an expression cassette containing a yeast promoter and transcriptional termination sequences and (ii) transformation and stable maintenance of this DNA in the fusion host.[14] The transformation process is highly dependent on the yeast strain and detailed studies should be conducted in order to achieve high-efficiency transformation.

This system is extensively used for studying biological processes in higher eukaryotes and also allows replication of eukaryotic viruses. The first eukaryotic virus for which replication and genome encapsidatation was conducted in *S. cerevisiae* was brome mosaic virus (BMV), a positive strand RNA [(+)RNA] virus that infects plants.[18,19] The BMV VLPs were subsequently purified for structure-to-function studies using cryo-electron microscopy (cryo-EM) studies.[19] Other (+)RNA viruses that have been successfully replicated in *S. cerevisiae* include the plant viruses tomato bushy stunt virus and carnation

Italian ringspot virus and animal viruses Flock House virus (FHV) and Nodamura virus.[20] Human papillomavirus-16 (HPV-16) VLPs have also been successfully expressed in the yeast system[21] in addition to the bovine papillomavirus-1 (BPV-1).[22,23] The yeast virus L-A was isolated and purified from *S. cerevisiae* and the structure was solved to 3.4 Å resolution.[24]

Insect Cells

Originally isolated from the alfalfa looper (*Autographa californica*) insect, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the most widely used and best characterized baculovirus for recombinant gene expression (a recent review on baculovirus molecular biology is available[25]). The rather large genome ($\sim$134 kbp[26]) can stably accommodate an insertion of $\sim$38kb,[27] making expression of large genes possible. This virus is also known to infect several other insect species including *Spodoptera frugiperda*. The most commonly used insect host cell lines, Sf9 and Sf21AE, are derived from *S. frugiperda* pupal ovarian tissue[28] and the BTI-Tn-5B1-4 line, also known as 'High 5 cells', derived from *Trichoplusia ni* egg cell homogenates.[29] The wt nucleopolyhedrovirus (NPV) produces small inclusion bodies composed of a polyhedron protein which allows for the encapsulation of many virions into a crystalline protein matrix. This protein is expressed in the very late phase of gene expression and is controlled by a very strong promoter, the polydron promoter (a review on baculovirus late expression factors is available[30]). The baculovirus expression vector system (BEVS)[31,32] takes advantage of this very strong polyhedron promoter to drive foreign protein expression. It has also been shown that the non-structural p10 protein is expressed at similar levels in the same very late phase of expression. Both proteins have been shown to be non-essential in the production of baculovirus particles,[33,34] making the replacement of their open reading frame (ORF) ideal for use in foreign gene expression.

The coupling of the very strong polyhedron promoter with a foreign gene-coding region results is the production of high levels of recombinant protein ($\sim$5–200 mg L^{-1}) in a relatively short amount of time using the BEVS. Since the baculovirus genome is generally considered too large to insert the foreign gene of choice by direct ligation, transfer vectors are used. There are many different vectors available for gene insertion, which are variants of a basic design (a review appeared recently[35]). These offer single gene, multiple genes and fusion gene expression. Multiple copies of the promoter can also be engineered into BEVS for the expression of multiple recombinant proteins concurrently in infected cells,[36,37] which permits the assembly of structures that are made up of heterologous proteins, such as viruses.

Advances in experimental design such as a wide variety of transfer vectors, simplified recombinant virus isolation and quantification methods, advances in cell culture technology and commercial availability of reagents have led to the increased use of BEVS for recombinant viral capsid protein production. Belyaev and Roy[37] were able to construct a multiple gene transfer vector which