Bombyx mori nucleopolyhedrovirus: Molecular biology and biotechnological applications for large-scale synthesis of recombinant proteins

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Bombyx mori* nucleopolyhedrosis virus (BmNPV), a natural pathogen of great economic significance in sericulture, has been exploited to generate recombinant baculoviruses harbouring genes of choice and achieve high level expression of cloned foreign genes. The BmNPV-based expression system offers the advantage of using the larval host rather than the insect-derived cell lines for economic large-scale synthesis of biomolecules. Since commercial rearing of silkworms is routinely practised, a part of the silkworm stocks can be diverted to produce biomolecules other than silk and the more sophisticated tissue culture methodologies can be dispensed with on an industrial scale. Nonetheless, the BmNPV-based expression considerably lags behind the AcMNPV system in vogue today, in terms of the choice of expression-optimized cloning vectors and easy recombinant generation kits that are commercially available. This review provides a brief outline of the current state of knowledge on the basic biology and genomics of BmNPV and how the virus has been made use of to produce biomolecules through its larval host, the mulberry silkworm. Since hyper-transcription from the viral very late gene promoters forms the basis of high level expression through recombinant baculoviruses, some aspects of the viral gene transcription and the role of late gene expression factors have also been included in the review.

BOMBYX *mori* nucleopolyhedrovirus (BmNPV, formerly known as *Bombyx mori* nuclear polyhedrosis virus), is a natural pathogen of the mulberry silkworm *Bombyx mori*, and is of great economic concern to the silk farmer. Since there are no specific preventive measures for the occurrence and spread of BmNPV infection other than sanitized breeding and rearing methods, the only commercial practice today is to discard large stocks of worms in case of infection. Although sensitive diagnostic kits for early detection of BmNPV have been developed, they are not extensively used on a commercial scale. Even if the infection is detected, in the absence of any remedial meas-

ures for the containment of virus infection, the only solution at present is to discard whole rearing trays containing thousands of larvae to avoid the spread of the disease. Early detection of virus infection, however, permits early discards without going through prolonged rearing procedures and the consequent higher economic loss. The multivoltine, native Indian races of *B. mori* (e.g. Pure Mysore and Nistari strains) in general, are more resistant to nucleopolyhedrovirus (NPV) infections compared to the exotic, high yielding bivoltine races. Although the genetic basis of susceptibility/resistance to viral infections in *B. mori* is not unequivocally established so far, engineering virus resistance to the more susceptible high yielders through genetic crossbreeding and possibly through transgenesis is of prime economic significance.

BmNPV belongs to the family of Baculoviridae, the largest and the most common among the seven families of viruses that infect arthropods. The baculoviruses have been used as biopesticides for insect control in early days, due to their narrow host range. Subsequently, the baculovirus based expression vector system (BEVS) has gained prominence for over-expression of cloned foreign genes in insect cells and larval caterpillars. Most eukaryotic proteins synthesized in insect cells are post-translationally processed in a manner similar to the native proteins and are biologically active. The molecular biology of baculoviruses[1–3] and their use as expression vectors[4–7] have been extensively reviewed in the past. The protocols for manipulating baculoviruses for recombinant protein expression are also available as laboratory manuals[8–12]. The majority of information has been generated through *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV), which is now considered as the prototype baculovirus. BmNPV is very closely related to AcMNPV and shares a significant level of homology at the genomic level.

Life cycle

A characteristic feature of the NPV infection is the production of crystalline proteinaceous structures called occlusion bodies (OB) or polyhedra, in which several

virions are embedded and thus protected from environmental factors such as UV light, desiccation, proteases and nucleases. The two morphological subgroups within the NPVs are the single nucleocapsid NPV (Type species: BmNPV) in which only one nucleocapsid is present per envelope, and the multinucleocapsid NPVs (Type species: AcMNPV) in which several (1–17) nucleocapsids are packaged per envelope.

Baculoviruses replicate in the nuclei of infected cells. The virus lifecycle is characterized by the production of two structurally and morphologically distinct types of virions, the occluded or polyhedra-derived virion (PDV) and the extracellular or budded virion (BV). The PDV is responsible for natural infection as the insect larvae ingest OBs along with food. The alkaline environment in the insect midgut dissolves the polyhedra and releases the virions. The liberated PDVs infect midgut epithelial cells by a receptor-mediated membrane fusion[13]. These infected cells produce the budded virions, which are also referred to as extracellular virus and are needed for lateral propagation of infection within the larvae via haemolymph and tracheae. The BVs infect many tissues in the larvae including fat bodies, ovaries and most endothelial cells[14–16]. Some tissues, however, escape infection. For instance, BmNPV does not infect the silk glands of *B. mori*, which constitute a major component of the larval body[17,18]. The BVs enter the cell through receptor-mediated endocytosis[19,20]. Following penetration of the plasma membrane, the nucleocapsid moves towards the cell nucleus by a process that requires actin-microfilaments. At the nucleus, the viral nucleocapsid is uncoated and the DNA gets released. The nucleus becomes enlarged and a distinct electron dense granular structure, called virogenic stroma is formed. This structure gets associated with nuclear matrix and forms the site of nucleocapsid assembly. Viral transcription and replication take place in this region. A typical electron micrograph of BmN cells infected with BmNPV is presented in Figure 1.

Following virus replication, by about 12 h post-infection (hpi), the progeny BVs are produced and released into the extracellular compartment. Soon after, mature PDVs (surrounded by an envelope) get packaged into occlusion bodies. Production of a large number of OBs results from the hyper-expression of polyhedrin (*polh*). The infected larvae continue to feed till large amounts of OBs accumulate. Eventually they stop feeding and undergo several physiological changes. The cuticle melanises, musculature becomes flaccid and the larva liquifies. Larval disintegration results in the release of polyhedra into the environment and the subsequent spread of virus infection.

The occlusion bodies stabilize the virions by allowing them to remain viable for long periods in the environment. In addition to baculoviruses, insect viruses from both the Reoviridae (e.g. cytoplasmic polyhedrosis
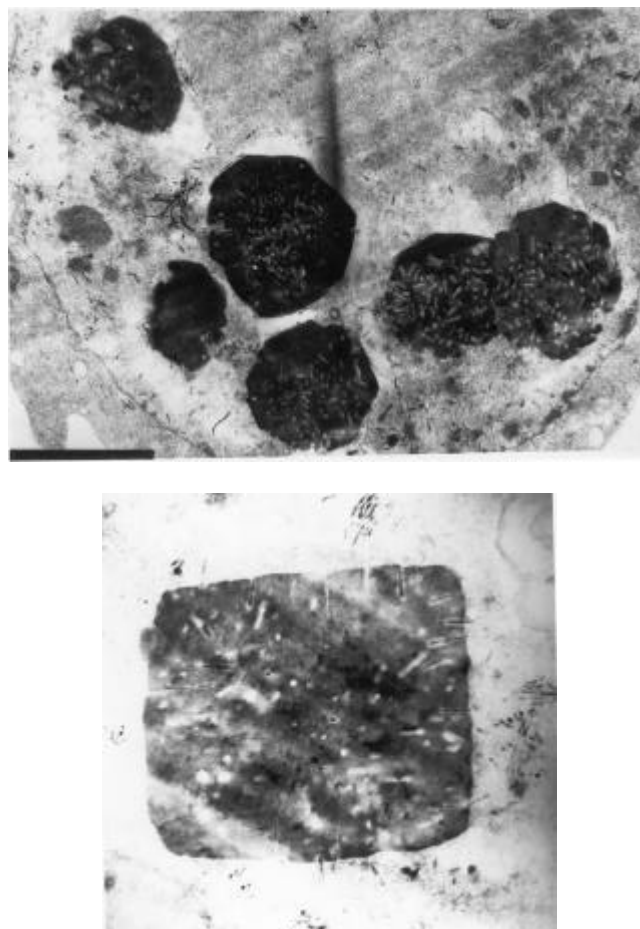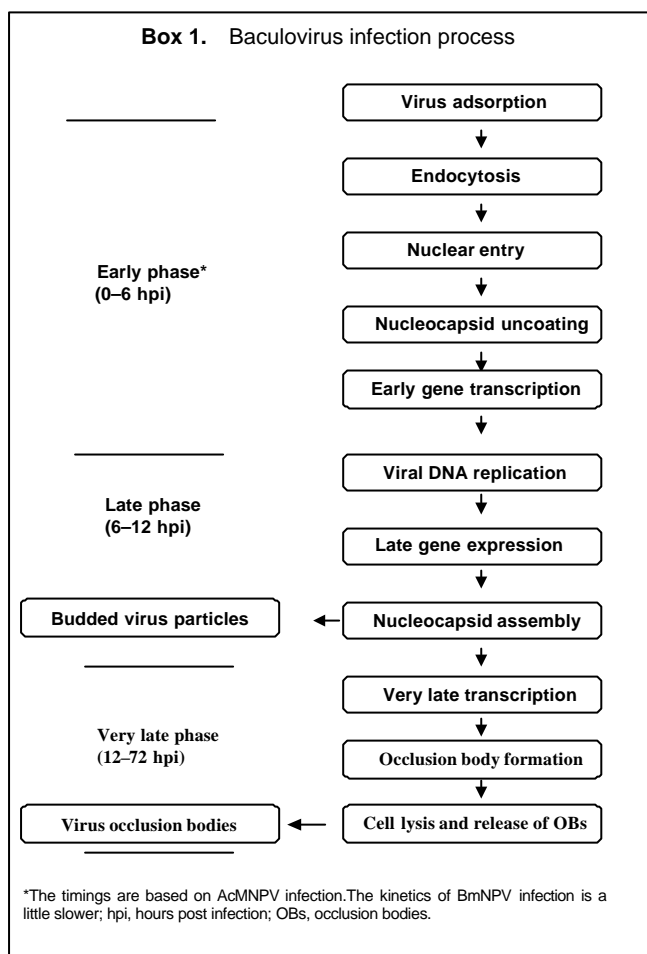

Figure 1. BmNPV infection of BmN cells. Transmission electron micrograph of BmN cells infected with BmNPV (60 h post infection), sectioned and viewed after staining with uranyl acetate and lead citrate is shown. The top panel shows the common features of a baculovirus infected cell. Occlusion bodies (OB) containing polyhedra-derived virus (PDV) are present in the nuclei. Replication and assembly of viral nucleocapsids occur in the nucleus in association with the virogenic stroma (VS). The budded viruses (BV) are produced in the nucleus and they bud out through plasma membrane. The PDV acquires an inner membrane before being packaged into OBs. The bottom panel shows the close up picture of BmNPV occlusion body.

viruses[21]) and the Poxviridae (e.g. entomopox viruses[22]) occlude their virions. Occlusion in these virus groups is also similar to that of baculoviruses in that the occlusion body protein gene is highly expressed and the occluded virions are released by the high pH encountered in the midgut of the susceptible insect. However, there exists no amino acid sequence or functional identity between any of the occlusion body proteins from these different virus families.

Baculovirus lifecycle *in vitro*, in the tissue culture system, can be divided into three phases, viz. early, late and very late (see Box 1). The kinetics of the infection process and the molecular events occurring during infection have been characterized extensively using AcMNPV and the host cell line, IPLB-*Sf*21 derived from *Spodop-*

Box 1. Baculovirus infection process

Early phase*
(0–6 hpi)

Virus adsorption
↓
Endocytosis
↓
Nuclear entry
↓
Nucleocapsid uncoating
↓
Early gene transcription
↓

Late phase
(6–12 hpi)

Viral DNA replication
↓
Late gene expression
↓
Nucleocapsid assembly → Budded virus particles
↓

Very late phase
(12–72 hpi)

Very late transcription
↓
Occlusion body formation
↓
Cell lysis and release of OBs → Virus occlusion bodies

*The timings are based on AcMNPV infection.The kinetics of BmNPV infection is a little slower; hpi, hours post infection; OBs, occlusion bodies.

tera frugiperda. BmNPV follows essentially the same pattern but the growth kinetics is a little slower.

Genomic DNA sequence of BmNPV

The complete nucleotide sequences of the baculoviruses AcMNPV, BmNPV, *Orgyia pseudotsugata* multinucleocapsid nucleopolyhedrovirus (OpMNPV), *Lymantria dispar* multinucleocapsid nucleopolyhedrovirus (LdMNPV) and *Spodoptera exigua* multinucleocapsid nucleopolyhedrovirus (SeMNPV) are currently available[23–27]. BmNPV shares a high degree of homology to AcMNPV genome whereas the OpMNPV and LdNPV genomic sequences differ markedly. The primary sequence of BmNPV has been analysed on the same lines as AcMNPV, to predict the potential protein-coding regions, gene organization, the sites for initiation of viral DNA replication and the presence of regulatory elements. The BmNPV genome is shorter by 5481 bp compared to the AcMNPV. The genomic organization of BmNPV is shown in Figure 2.

Most of the ORFs in BmNPV are present at comparable locations and share about 90% identity to the AcMPNV genome. The subtle changes in sequences, however, are responsible for the morphological and kinetic differences between them, as well as the host specificity range. For instance, the species specificity of infection by AcMNPV has been attributed to the *dna-hel*[28,29]. AcMNPV does not replicate in *B. mori* derived Bm5 or BmN cells, but when a short sequence within AcMNPV ORF95 (encoding viral DNA helicase, p143) is replaced with a collinear region of BmNPV, it acquires the ability to replicate in Bm cells or *B. mori* larvae. Amino acid changes at positions 564 (serine to asparagine) and 577 (phenylalanine to leucine) were sufficient to extend the host range[29]. Recombination between AcMNPV and BmNPV could be achieved through coinfection of the viruses in the susceptible host cell line, to generate viruses with dual host range.

A notable feature of the genomic sequence is the presence of clustered *Eco*RI sites at five locations in the genome. These regions contain two to eight repeats of an imperfect palindrome with an *Eco*RI site at the centre of each palindrome. These regions being homologous to each other, are designated as homologous regions (hr). In AcMNPV there are nine such homologous regions, *hr*1, *hr*1a, *hr*2, *hr*2a, *hr*3, *hr*4a, *hr*4b, *hr*4c and *hr*5. They are 200 to 1000 bp long and are located 15 to 55 kb apart in the 134 kb genome. Each repeating unit is usually 75 bp long with the *Eco*RI site at its core flanked by sequences, forming a 29 bp palindrome motif. The *hr*4 from AcMNPV is subdivided into left and right halves[30].

The BmNPV genome also possesses seven *hrs* (*hr*1, *hr*2-left, *hr*2-right, *hr*3, *hr*4-left, *hr*4-right and *hr*5) found at positions similar to those in AcMNPV. The consensus sequence of the BmNPV *hrs* shows 95% homology to that in AcMNPV. Nucleotide sequence analysis of the BmNPV *hr*2, which is subdivided into left and right halves, suggests that it could have evolved from the ancesteral AcMNPV analogue by inversion, cleavage and ligation. The polarities of the BmNPV and AcMNPV *hrs* are conserved except for that of *hr*4-left[31]. The OpMNPV genome also harbours *hrs* but their locations are not comparable to the *hrs* of AcMNPV or BmNPV. Baculovirus *hrs* are involved in viral gene expression and in replication[32]. Transient expression assays showed that the *hrs* activate the baculovirus early genes *38K*, *ie*-2 and *p35* when placed either upstream or downstream to these genes. Similarly *hr*5 can activate gene expression from host promoters like *actin* 5C (ref. 33). This enhancement of gene expression is independent of viral infection but the virally encoded protein IE-1 stimulates the expression over 1000 fold. *Hr*1 has been reported to stimulate *polh* gene expression[34]. The *hrs* can also serve as origins of DNA replication[35–37].

A few ORFs, such as ORF 603 in the *polh* locus and *pcna* from the *lef*8 region present in AcMNPV, are absent from BmNPV whereas some others like *bro* (*baculovirus related ORFs*) are repeated more frequently. The *bros* constitute a unique and highly conserved multigene family, first reported from genome analysis of LdMNPV
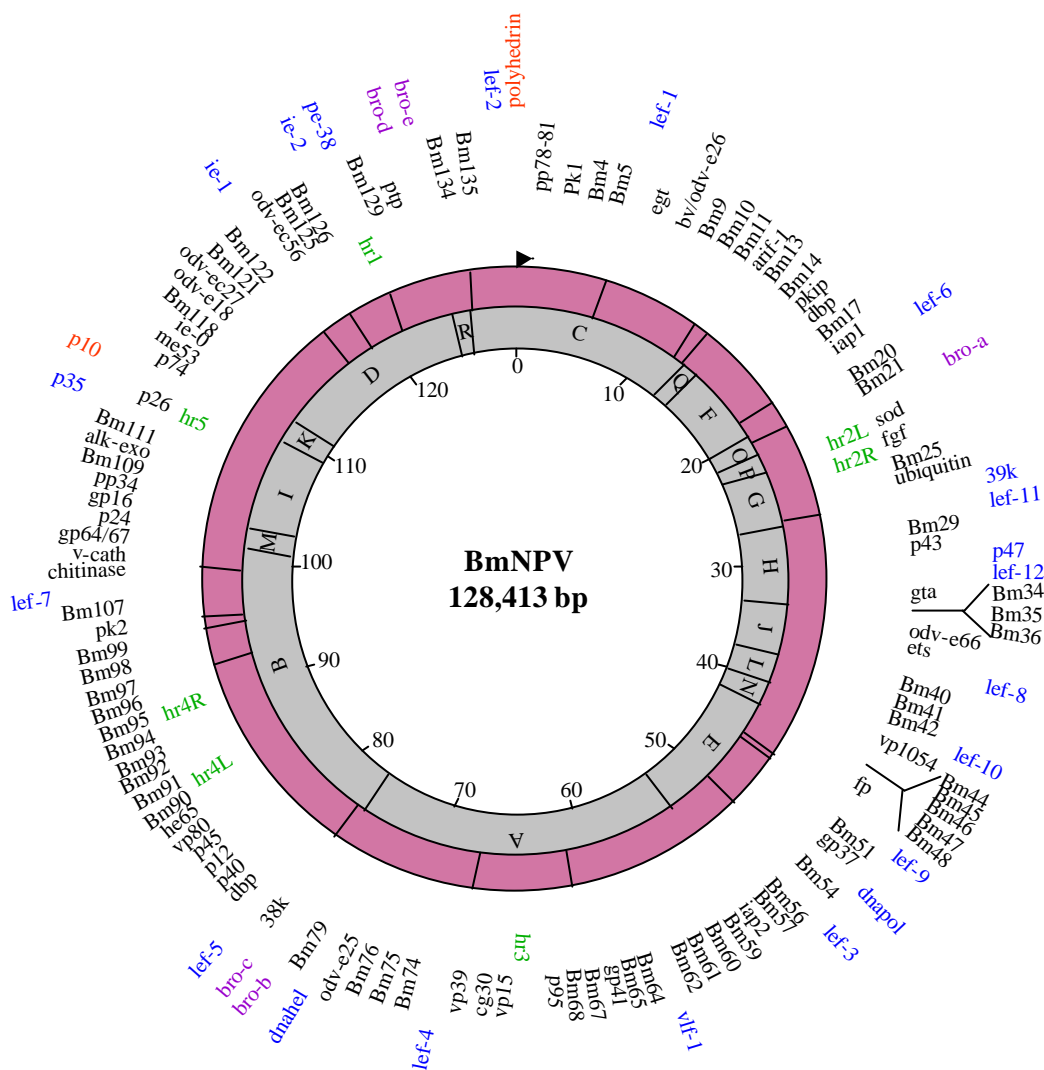
Figure 2. Genomic organization of BmNPV. BmNPV genome is a covalently closed circular DNA of 128,413 bp. The restriction maps for the enzymes *Pst*I and *Xho*I are shown (pink and grey, respectively) and the alphabets (capitals) mark the different fragments. The approximate locations of the seven *hr* sequences (*hr*1, *hr*2L, *hr*2R, *hr*3, *hr*4L, *hr*4R and *hr*5), which serve as potential origins of replication and/or enhancers for late gene expression, are marked in green. The *late gene expression factors* (*lef*s) involved in late gene transcription are marked in blue, and the *polyhedrin* and *p10* genes which are mostly extensively exploited for high level expression of recombinant proteins are marked in red. All other markers are designated as the ORF numbers with a prefix of *Bm* (e.g. *Bm*4, *Bm*5) or based on their counterparts from AcMNPV (e.g. *gp64*, *vp80*). The 5 *bro* genes (baculovirus related ORFs) are also marked in purple. This circular map has been constructed based on the information in ref. 24. For other details of the genomic markers and functions, see ref. 24.

(which had 16 copies) homologous to the single copy ORF2 in AcMNPV. There are 5 copies of *bro* in BmNPV and 3 in OpMNPV. They are generally present as contiguous clusters. The *Ac-bro* (ORF2), *Bm-bro* d and *Ld-bro* n are the most highly conserved ones (82% identity in amino acid sequence) and many have similar functions in the infection cycle. All the 5 *Bm-bro* genes (*bro* a, b, c, d and e) are expressed as delayed early genes, and *bro*-d and e are located adjacent to each other in a head to tail arrangement[38]. BmNPV mutants lacking *bro* a, b, c, and e were viable whereas a null mutant of a *bro* d (equivalent of the single copy Ac-*bro*) was non-viable. It is speculated that both *bro* a and c products may function as DNA-binding proteins that influence DNA replication and/or trancription[39]. The BmNPV transregulator IE-1 was required for the activation of *bro* c promoter[40].

Although nearly 135 genes (ORFs) are present in BmNPV, so far only a limited number has been characterized (listed in Table 1)[41–58]. However, most of the functions have been deduced based on the AcMNPV counterparts. The viral very late genes, both *polh* and *p10* whose strong promoters have been mostly exploited for recombinant protein expression, have been studied in some detail[41–43]. A striking difference is that the BmNPV

p10 encodes a 7.5 kDa protein due to the presence of a nonsense mutation arising from the deletion of an 'A' residue at the 3′ region[44].

Gene expression patterns

Gene expression pattern following baculovirus infection is regulated in a cascade fashion, where the activation of each set of genes relies on the synthesis of proteins from previous class. Based on this temporal regulation, the baculovirus genes are grouped into three classes: early, late and very late. Although most baculovirus genes can be placed into one of the above classes, some are transcribed in more than one phase. The early genes are transcribed prior to viral DNA replication and are subdivided into immediate early and delayed early class, whereas the late and very late genes are activated during or after viral DNA replication. The late genes are maximally active between 12 and 24 hpi. The very late genes are hyper-expressed following activation of late genes and remain active well after late gene expression has diminished. The early genes generally encode proteins of regulatory functions, transcription, replication, and modification of host processes. Late proteins include those involved in regulation of late and very late gene expression and the viral structural proteins. Very late proteins are those involved in the processes of occlusion and cell lysis.

Early genes are transcribed by the host RNA polymerase II. Many early promoters contain a functional TATA box[59] typical of most RNA polymerase II promoters of higher eukaryotes. Immediate early gene promoters are accurately transcribed by nuclear extracts prepared from uninfected host cells[60]. In addition to the TATA box, many baculovirus early promoters contain a conserved sequence CAGT, at or near the transcription start site[61–63]. However, there are exceptions. For instance, the transcription of DNA *pol* from both BmNPV and AcMNPV initiates from a G/C rich sequence (5′-GCG-TGCT-3′ and 5′-AGAGCGT-3′) with no apparent TATA box in the vicinity[49]. Likewise, the AcMNPV *lef*4 transcript initiates from a G/C rich sequence and the Ac-*iap* transcript initiates from the sequence GAGTTGT. In addition to the basal promoter elements, many enhancer elements have also been identified in early promoters.

The early genes are subdivided into two groups, immediate early (*ie*) and delayed early (*de*). The former ones are fully active in the absence of viral factors whereas the *de* promoter requires one or more IE proteins for full activation. Most of the IE proteins are involved in the regulation of viral transcription. The deletion of *ie*-2 from BmNPV reduces viral DNA replication by 2 fold in BmN cells[64].

Virus infection is known to trigger the host cell death programme, apoptosis. AcMNPV however, evades this by encoding an inhibitor of apoptosis, p35, which is an inhibitor for a family of host cysteine proteases[65]. The BmNPV homologue of p35, with 90% identity (at the amino acid level) to the AcMNPV counterpart has been identified[46]. However, the two proteins seem to differ in their apoptotic activity[66].

Late and very late transcription is dependent on early viral gene expression and viral DNA replication[67,68]. The late and very late promoters are unusual with respect to their structure and location. They differ from most RNA polymerase II promoters and do not contain DNA elements such as TATA box. The primary determinant for the late and very late promoter activity is the pentanucleotide A/G/TTAAG, which is located at the transcription start point of all known late and very late genes[2,69]. Late and very late promoters are distinguished by their relative activities during late and very late phases. The promoters of *polh* and *p10* belonging to the very late class, exhibit low activity during the late phase (6–18 hpi) but become highly active at 18hpi. By 24 to 48 hpi approximately 20% of the total polyadenylated RNA in the cell is *polh* mRNA. The *polh* promoter is relatively well conserved between distantly related baculoviruses[2] and is marked by the presence of a 12nt consensus sequence spanning the transcription start site

Table 1. Genes characterized from BmNPV

Gene	Function	Reference
polh	Major viral occlusion body protein	41
p10	Viral occlusion body protein forming fibrillar structures	42 43, 44
p39	Structural polypeptide	45
cg30	Putative transcriptional regulator, (harbours a RING finger like motif, a leucine zipper and an acidic domain)	45
p35	Anti-apoptotic protein	46
p40	Virion-specific occlusion polypeptide	47
lef-3	ss-DNA binding and helix destabilizing activity	48
p143/dnahel	Host range determining factor	28
dnapol	DNA polymerase	49
lef-8	Virus replication *in vivo* and *in vitro*	50
lef-2	Virus replication and late gene expression	51
hr5	Non-essential for virus replication	31
hr3	Transcriptional enhancer	33
bro-a, b, c, d, e	DNA-binding proteins	39
dbp	ss-DNA-binding protein and colocalizes with IE-1 and LEF-3 in viral replication machinery	52 53
fp25	Causes few polyhedra phenotype and implicated in post-mortem host degradation	54
p95	Transcriptional regulator (harbours 2 Zn finger motifs and a proline-rich region)	55
ie-1	Transactivator protein essential for initiation of viral DNA replication, transcription from *bro* c promoter	56 39
v-cath	Encodes a cysteine proteinase involved in aborting host protein synthesis machinery, horizontal transmission of infection	57
p6.9	Basic DNA binding protein	58

(A/T)(A/G) TAAGNA(T/A/C)(T/A)T. This consensus is also present in the very late *p10* gene promoters.

The *polh* promoter of AcMNPV has been characterized most extensively by deletion[69] and linker-scan mutational analyses[70,71]. The transcription initiation site is within the highly conserved sequence TAAATAAGTATT. The minimal essential *polh* promoter has been defined as a 69 bp stretch (–1 to –69 nt with respect to + 1 ATG) to confer hyper expression at very late periods. The TAAG portion of this sequence is absolutely essential for transcription initiation to occur because linker scan substitutions affecting the TAAG site decreased reporter gene expression approximately 2000-fold and lowered the steady state levels of RNA to undetectable levels. Sequences in the leader region between TAAG and the start codon also have a strong influence on *polh* transcription. Sequential deletion in the *polh* leader sequence upstream from the ATG adversely affects the reporter gene expression[72]. In *polh* 5′ upstream sequences of BmNPV also, transcription enhancer elements that stimulate expression from *polh* promoter are present[73].

Sequences proximal to the *polh* start codon (up to + 35 nt) substantially increase the level of expression of foreign genes in recombinant baculovirus vectors in AcMNPV, possibly by increasing the stability of mRNA[5]. In BmNPV, the sequence till + 5 nt from the first ATG of polyhedrin was sufficient to direct maximal expression of cloned foreign genes[74].

The virus-encoded genes responsible for mediating the late gene transcription have been identified in a transient expression system utilizing a mixture of successively smaller fragments of the baculovirus genome that are able to activate the late and very late promoters[75,76]. Nineteen late gene expression factors (*lefs*) have been identified to be necessary and sufficient to mediate late gene expression from late and very late promoters. These genes could have directly activated late/very late gene transcription or exerted indirect effect by stimulating early gene transcription or through mediating DNA replication. Nine of these (*ie*-1, *ie*-2, *lef*-1, *lef*-2, *lef*-3, *lef*-7, *p143*, *dnapol* and *p35*) are involved in *hr*2-based DNA replication whereas the products of the other nine genes (*lef*-4, *lef*-5, *lef*-6, *lef*-8, *lef*-9, *lef*-10, *lef*-11, *39k* and *p47*) affect the steady state levels of reporter gene transcripts and are thus likely to be involved in some aspects of transcription, transcript processing or stability[68]. More recently, however, a complex of *lef*-4, *lef*-8, *lef*-9 and *p47* encoded proteins from AcMNPV has been shown to initiate transcription from *polh* promoter *in vitro*[77].

Most of the *lefs* have been identified only by transient expression studies and their individual functions are not yet delineated. The available information on the function of *lefs* has been summarized in Table 2. The role of *lefs* in virus multiplication and late gene expression *in vivo* in BmNPV has been investigated by deleting each *lef* homologue from the genome[64]. Of the 19 *lefs*, deletion mutants could be isolated only for 4 (*39k*, *ie*-2, *lef*-7 and *p35*). Deletion mutants for the other 15 could not be generated probably because they are essential for virus multiplication. The *lef2* from BmNPV encodes a 23 kDa, dual function protein, which plays a role in both viral DNA replication and late gene expression[51]. The function in

Table 2. Late gene expression factors from BmNPV[@]

Gene	ORF (bp)	Size (kDa)	Identity* aa (%)	Promoter	Homology/ motifs	Function
lef-2	630	23.6	96	E/L	Cys-rich	Replication/transcription
lef-1	810	31.0	96	E/L	Primase	Replication
lef-6	519	20.2	93	–	–	LEF
39k	831	31.3	92	E/L	Basic, DNA binding	LEF, Phosphoprotein (ne)
lef-11	336	13.0	97	e	–	LEF
p47	1197	47.1	98	e/E	–	DNA binding, LEF
lef-8	2631	101.6	98	–	RNApol*b*	Transcription
lef-10	417	16.7	96	L	–	LEF
lef-9	1470	56.2	98	–	RNApol*b*	Transcription
dnapol	2958	114.3	96	e/E	DNA pol	Replication
lef-3	1155	44.7	92	E	SSB	Replication
vlf-1	1137	44.2	98	L	Integrase	Very late expression
lef-4	1395	53.8	97	E	Acidic domain	Transcription
dnahel	3666	143.5	96	E	Helicase	Replication, host specificity
lef-5	795	31.0	97	–	–	LEF
lef-7	636	24.9	88	E	SSB, UL 29(HSV)	Replication (ne)
p35	897	34.8	91	E/L	–	Block apoptosis (ne)
ie-1	1752	66.8	96	E	–	Transactivator
ie-2	1266	48.7	73	E/L	Zinc finger	Transactivator (ne)

E, early; L, late; e, enhancer in the near upstream region; ne, nonessential.
LEF, Late gene expression factor protein encoded by *lef* genes
([@]modified from ref. 64; *identity to AcMNPV counterparts).

late gene expression is possibly as a transcriptional coactivator (Sriram and Gopinathan, unpublished observations).

A viral gene for the 'burst' of very late gene transcription, *very late gene expression factor*-1 (*vlf*-1) has also been identified in AcMNPV and a mutation in the gene results in significant reduction in *polh* and *p10* mRNA levels[78]. The *vlf*-1 encoded protein shows considerable homology to the lambda phage integrase family and the mutation in any of the conserved amino acid residues adversely affects the virus production[79]. A counterpart for *vlf*-1 is present in BmNPV also.

A comparative analysis of gene expression patterns in BmN cells following BmNPV infection has been carried out recently[80]. The profiling of expressed sequence tags generated at different times following virus infection confirmed the predicted patterns. For instance, *ie*-0 and *ie*-2 transcripts could be detected in 2 h, whereas the transcripts of several genes associated with viral DNA replication and the *lef*s as well as structural genes like *gp64* were detected by 6 h. Interestingly, in this c-DNA approach, the expression profiling of several host genes, ranging from complete suppression to enhanced synthesis consequent to virus infection, could also be established.

BmNPV-based high level expression of cloned foreign genes

Baculovirus-based expression systems using recombinant insect viruses in insect cell lines have become the method of choice in recent years for generating biomolecules in large amounts for basic biological studies and biomedical purposes. Although over 500 baculoviruses are known, AcMNPV has been most thoroughly developed, followed by BmNPV for expressing foreign genes. More than a thousand genes, ranging from the components of basal transcription machinery such as TBP and transcription factors to viral coat and envelop proteins (e.g. HIV major surface glycoprotein, GP120 and envelope protein, GP41) have been cloned and expressed through recombinant AcMNPV and BmNPV. Baculovirus-based expression in insect cells provides an environment that is conducive to proper folding, disulfide bond formation, oligomerization and other post-translational modifications of the synthesized protein, all of which are essential to achieve biological activity.

Two desirable features of baculoviruses account for their success as expression vectors. First, the virus contains a number of nonessential genes that can be replaced by a foreign gene. Second, many viral genes, particularly the very late ones are under the control of strong promoters that allow abundant expression of the passenger recombinant gene. For high level expression of recombinant protein, most frequently the two very late genes, *polh* and *p10*, which are hyper-transcribed and nonessen-

tial for virus multiplication, are replaced by the foreign gene. This allelic replacement is achieved by homologous recombination between the wild type virus and a transfer vector harbouring the foreign gene. However, the baculovirus genome is too large to permit routine manipulations for insertion of foreign genes. Therefore, the generation of recombinant virus is achieved as a two-step process: (i) the construction of recombinant transfer vector harbouring the foreign gene of interest and (ii) the production of recombinant baculovirus by cotransfection of insect cells with wild-type viral DNA and the transfer vector.

The transfer vector is a bacterial plasmid carrying a portion of the baculovirus genome spanning a strong promoter to be used for foreign gene expression (e.g. *polh*, *p10*) and its 5′ upstream region as well as the downstream region flanking the termination sequences in order to facilitate recombination with the viral DNA. Transcriptional enhancer elements that stimulate expression from the *polh* promoter are also present in the 5′ flanking region[73]. A suitable, multiple cloning site (MCS) is located immediately downstream to the promoter so that the expression of the inserted foreign gene at the MCS is controlled by the viral promoter. The minimum lengths of the upstream and downstream sequences needed for optimal recombination are not yet fully characterized. The first generation transfer vectors were very large (more than 10 kb in size), and therefore, were limited in their capacity to accommodate large foreign genes or multiple genes as inserts. More recently the size of the vectors has been reduced to accommodate large or multiple foreign genes.

Both *polh* and *p10* promoters from AcMNPV are recognized efficiently as the homologous promoters in BmNPV-infected BmN cells[17]. The expression from *p10* promoter ranges from 20 to 60% of that from the *polh* promoter. The levels of expression from *polh* promoter, in general, are dependent on the position at which the recombinant gene is located. Thus, in BmNPV-infected BmN cells, the expression of the reporter gene from BmNPV *polh* promoter when located at –3 nt with respect to the +1 ATG was only 10–20% of the expression levels from the heterologous AcMNPV *polh* promoter, in which the gene was located at + 35 nt (Sehrawat and Gopinathan, unpublished results). Many transfer plasmids for multiple gene expression and for expression under alternate promoters are currently available for AcMNPV. The presence of purification tags (e.g. glutathione-S-transferase fusion or affinity His tag) and secretory signals at the cloning site are important new additions to BEV technology.

Traditionally, recombination between the transfer plasmid and viral genomic DNA is carried out in insect host cells following transfection. The plasmid and viral DNAs are cotransfected by calcium phosphate-mediated DNA uptake or lipofection. Alternately, the insect cells are

transfected with the plasmid DNA and followed by viral infection. After 48–72 h of transfection/infection, viral progenies are screened for recombinants wherein a segment of the viral genome is replaced by the region harboured in the transfer vector plasmid. The recombinant viruses are isolated by several rounds of plaque purification.

The type of selection method used for isolating recombinant virus depends upon the parental virus used, the gene being replaced in the virus and the selection marker available in the transfer vector. If the foreign gene is inserted at *polh* locus, the recombinant virus will demonstrate an occ⁻ phenotype and the screening procedures depend on the tedious selection for occ⁻ plaques. Typically, only 0.1–1% of the progeny viruses are recombinants. Hence, a large number of plaques need to be screened. However, many improvements have occurred in achieving recombination and plaque selection in the last few years, mostly in the AcMNPV system. If linearized viral DNA is used in the cotransfection step instead of the native viral DNA (which is circular), the proportion of recombinant viruses in the progeny increases to about 25% (ref. 81). A modification of this approach[82] involves the removal of a portion of the genome encompassing parts of an essential virus gene (ORF 1629, located downstream of *polh*), by restriction enzymes. Under these conditions more than 85–95% efficiency recombinant virus generation is achieved. Direct ligation of foreign gene into baculovirus vectors is another attractive way for generating recombinant viruses[83]. Modified AcMNPV DNA, with a multiple cloning site composed of unique restriction sites (e.g. *Bsu*361, *Sse*83871 and *Srf*I) has been engineered to facilitate direct ligation of *Eco*RI, *Pst*I and blunt-ended DNA fragments[84].

The other modifications of baculovirus recombinant selection involve the use of yeast and bacterial cells where the entire baculovirus genome is maintained. The AcMNPV genome has been reconstituted as a replicon to propagate in the yeast, *S. cerevisiae* by inserting a yeast autonomously replicating sequence (ARS) at the *polh* locus together with a centromere sequence to ensure stable low copy number segregation of the viral genome and serve as a suitable selection marker[85]. This allows easier manipulation of the viral genome in yeast and more straightforward selection procedures. Similarly the AcMNPV genome has also been reconstituted as a replicon in *E. coli*[86], by inserting the origin of replication from the mini-F replicon at the viral *polh* locus along with *kan*ʳ and *lacZ* as selection markers. This baculoviral DNA, which multiplies in both insect cells and bacteria, is called 'bacmid'. The insertion of foreign gene is achieved by a site-specific recombination mediated by Tn10 transposon at the *lacZ* locus. The transposase enzyme is encoded by a helper plasmid. The recombinant bacmid, thus can be selected by a simple blue-white selection in *E. coli*. The recombinant baculoviral DNA isolated from the bacterial host is directly transfected into the insect cells to generate the recombinant virus with a high proportion (almost 100%) of recombinants.

Making use of the above principles, several high efficiency recombinant generation kits are commercially available for AcMNPV-based expression and they are marketed under various commercial names such as 'Baculogold', 'Bacpac', 'Bac-to-Bac', etc. These kits provide simple and direct selection for recombinant plaque selection and purification in a week's time, but most of these technologies are restricted to the AcMNPV system. The BmNPV-based expression system mainly relies on the tedious occ⁻ plaque selection methods. However, the successful use of recombinant BmNPV harbouring *luc* or *gfp* as a parent for recombinant generation has somewhat simplified the recombinant selection that can be achieved through scoring for loss of luminescence or gfp fluorescence[17,18].

The cell lines supporting AcMNPV replication (*Sf*9, *Sf*21 and TN386) are superior in growth characteristics and expression levels to cell lines supporting BmNPV replication (BmN and Bm5). Besides, the range of transfer plasmid vectors and parent viruses available for the AcMNPV-based expression system is much more than that of BmNPV system. Moreover, a variety of late and very late promoters with protein purification tags (GST, His) and secretory signals, as well as methodologies for rapid recombinant selection, are available for the AcMNPV-based system. Nonetheless, the BmNPV-based expression system offers the advantage of economically viable, over-production of recombinant proteins in insect larvae rather than in cell cultures. The larval host of BmNPV, the silkworm *Bombyx mori* is 10 times larger than the larval hosts of AcMNPV and can be readily reared in the laboratory on mulberry leaves or a synthetic diet. Therefore, once the recombinant virus is generated in cell culture, the larval caterpillar can be exploited for the production of the recombinant protein in lieu of cell cultures. Thus, the use of a BmNPV-based larval expression offers an attractive, more economic protein production system, particularly for proteins that are secreted in the hemolymph.

The exploitation of BmNPV and the use of *B. mori* larvae for the first time, for the production of recombinant interferon was reported about 16 years ago[87]. Subsequently our own group here has improved the transfection efficiency of BmN cells by employing lipofection, which facilitated the recombinant generation[88]. Very high levels of expression of *luciferase* through recombinant BmNPV in the larval caterpillar (10 mg recombinant protein per larva) were achieved, which resulted in the generation of 'Glowing silkworms' emanating significant luminescence on administration of the substrate luciferin (Figure 3 *a*). We have also demonstrated that the larval expression succesfully handles multiple intron splicing, albeit with limited efficiency[89].
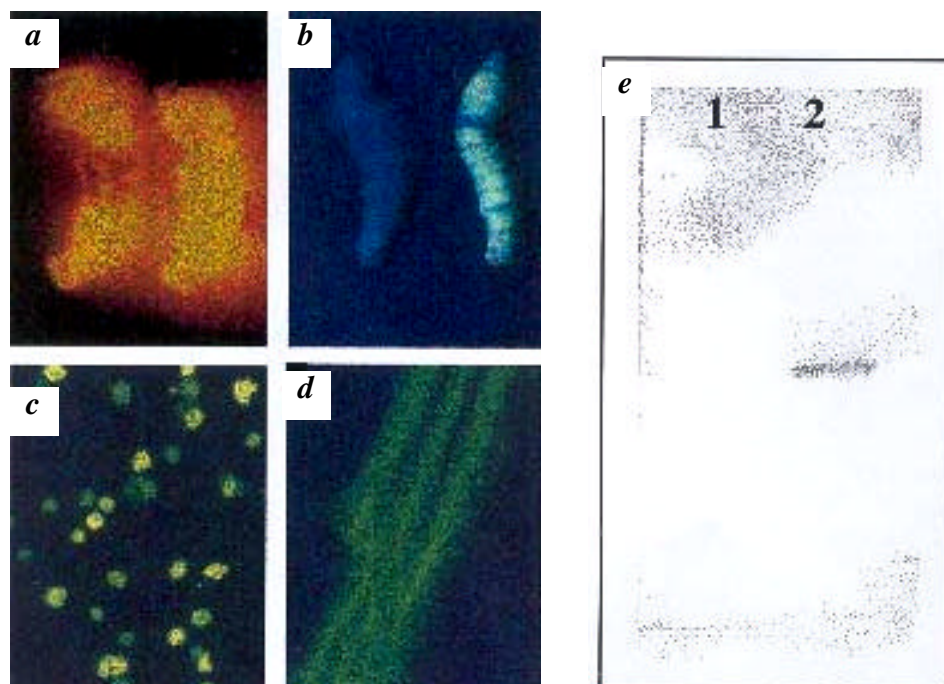
Figure 3. Expression of recombinant proteins in *B. mori* larvae. *a*, Silkworm larvae expressing high levels of luciferase on infection with recombinant BmNPV harbouring firefly *luciferase* gene, emanates significant amounts of luminescence on administration of the substrate luciferin. *b*, *B. mori* larvae infected with recombinant BmNPV harbouring the *gfp* gene under the *polh* promoter expressing green fluorescent protein (larvae on the right). Control larvae infected with wild type BmNPV is shown on the left. *c* and *d*, BmN cells in culture and the trachea from *B. mori* larvae infected with recombinant BmNPV harbouring *gfp* showing high levels of expression of the green flourescent protein. *e*, Expression of recombinant antigen ORF3 from Hepatitis E virus. Western blot of the fat body proteins from recombinant BmNPV infected *B. mori* larvae using the polyclonal antibodies to ORF3, showing the single band of Hepatitis E-ORF3.

The complex eukaryotic gene *hGH*, encoding the human growth hormone harbours 4 introns and 5 exons. This gene transcript was properly spliced and the translated product was secreted into the insect circulation system. The hGH from the haemolymph could be readily purified in a single-step immunoaffinity chromatography and the insect-synthesized protein was indistinguishable from the authentic hGH in both radioimmunoassay and radioreceptor assay[89]. However, the expression levels of *hGH* were much lower (100–150 μg per larva) compared to luciferase.

Subsequently, we have shown that the *polh* and *p10* promoter constructs from AcMNPV system can be successfully utilized to generate BmNPV recombinants through homologous recombination[17,18]. These results established that the wide variety of AcMNPV-based transfer vectors currently available can be utilized for generating BmNPV recombinants, despite the fact that *polh* promoter flanking sequences are not identical in these viruses. The generation of the recombinant BmNPV harbouring the *green fluorescent protein* gene (*gfp*) has opened up the possibility of using this tagged virus for pathological investigations on BmNPV infection in *B. mori* larvae, as it leaves the footprints of *gfp* expression (see Figure 3 *b–d*). The larvally expressed GFP, though a nonsecretory protein, could be readily isolated from the larval fat bodies. However, since the *B. mori* larvae have an open circulatory system and the fat bodies as well as most other tissues lie immersed in the haemolymph, substantial quantities of even non-secretory proteins leach out into the haemolymph. Therefore, the recovery of the larvally expressed recombinant proteins, even when they are nonsecretory in nature, can be readily achieved.

Our group has also expressed a viral antigen designated ORF3 from Hepatitis E virus. However, the expression levels in cell lines or larvae were very low and the expressed viral antigen could be detected only in Western blots (Figure 3 *e*). The reason for the very low levels of expression is not clear but the codon bias and codon usage patterns could be the main contributing factor.

A comprehensive but not necessarily complete list of the proteins expressed so far through recombinant BmNPV and *B. mori* derived cell lines or larvae is provided in Table 3.

Since the *polh* locus is mostly utilized for the insertion of foreign genes, the recombinant virus generated will have a *polh⁻* phenotype and the occlusion bodies are not formed at the culmination of infection. This ensures a built-in containment from inadvertent infection of other insect larvae because the non-occluded viruses do not survive long in the environment, and nor are they infec-

Table 3. Genes overexpressed in the BmNPV system

Gene	Reference
Human a-interferon	87
Polyhedrin-IGF II fusion protein	90
E2 ORF of BPV1	91
HPVtype 6b E2 gene product	91, 92
Trans-activator p40x of human T-cell leukemia virus type I	93
Hepatitis B virus surface antigen	94, 95
Human beta-interferon gene	96
Luciferase	88
Beet western yellows luteovirus capsid proteins	97
Human growth hormone	98, 89
Human granulocyte-M CSF	99
Human 17 b-hydroxysteroid dehydrogenase	100
Grass carp growth hormone	101
Green fluorescent protein	18
Canine parvovirus VP2	102
Human butyrylcholinesterase	103
Human EPO gene	104
Bovine interferon-gamma	105
Hepatitis E antigen, ORF 3	This review

tive to the larvae by the oral route. These nonoccluded viruses have to be injected into the haemolymph to initiate the infection. Therefore, biosafety is built into the system.

A double recombinant BmNPV harbouring two foreign genes at *polh* and *p10* loci would provide the advantage of simultaneous expression of proteins, which may be desirable at times.

Conclusions

It is evident that while considerable information is available on the molecular biology and application of AcMNPV as an expression system, the parallel system of BmNPV has lagged behind. The lack of availability of simplified recombinant selection procedures and a vast collection of optimized transfer vectors, as well as the 'more demanding' behaviour of the *B. mori* derived cell lines in culture, have been the major deterrents. However, the demonstration that heterologous AcMNPV-based vectors can be used to generate BmNPV recombinants has circumvented at least one of the problems. Further, the use of silkworm larvae, rather than *B. mori* derived cell lines for the high level expression of foreign proteins (once the recombinant virus is generated), confers a distinct advantage to the BmNPV system. Since commercial rearing of silkworms for the production of silk is widely practised and is inexpensive compared to the tissue culture methodologies, a small proportion of the silkworm rearing stocks may be channelled to produce, 'high value', 'low volume' biological products, utilizing the silkworm as a natural bioreactor. This technology offers tremendous economic potential to 'sericulture countries' like India.

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