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10. Baculovirus expression technology: Theory and application

Some History

I participated in a symposium on microbial pesticides organized by Lois Miller as part of a regional American Society for Microbiology meeting in Moscow, Idaho in mid-June, 1983. At that meeting she showed me some pictures of a baculovirus expression system — blue β-galactosidase plaques on *S. frugiperda* cells — developed in her laboratory by an undergraduate student, Greg Pennock. She indicated that she was using them as cover pictures for an issue of *Cell* to which she had submitted a manuscript describing this work. Later that summer, at the most riveting American Society for Virology workshop that I ever attended, she described this system as did Gale Smith, who showed baculovirus expression of an interferon. Gale Smith and Max Summers published their report later in the year (1). Lois Miller's report was rejected by *Cell,* thereby delaying publication until 1984 (2) and giving Max Summers and Gale Smith clear precedence with regard to this technology. In addition, their production of a practical manual detailing the use of the system (3) and distribution of its major components greatly facilitated its widespread application before it was commercially available. The development of this technology gave birth to a new era in baculovirology based on targeted gene recombination and has resulted directly or indirectly in much of the progress in understanding the biology of these viruses along with advances in many other biological systems dependent on protein expression technology. The application of this technology culminated with the licensing by the FDA of vaccines produced by recombinant baculovirus that are directed against human papilloma virus and influenza (4, 5). Veterinary vaccines against porcine circovirus and swine fever virus are also in use, reviewed in (6). The variations and modifications of the baculovirus expression system are not within the scope of this book and have been ably covered in several recent reviews (7). However, I will give a brief overview of the combination of biological features of insects and baculoviruses that have contributed to the evolution of a system of such utility for biotechnology. These are summarized in Table 1. I will then cover the major advances in the development of the bacmid based systems that has revolutionized baculovirus expression technology.

Table 1. Major factors influencing high levels of baculovirus very late gene transcription

	Factor	Result
1	Ability of virus to cause systemic infection	Allows virus to exploit insect synthetic systems, e.g., the fat body
2	Shut off of early baculovirus and host gene transcription	Makes host cell biosynthetic systems available for baculovirus gene expression

Table 1. continued from previous page.

	Factor	Result
3	Shut off of baculovirus late gene expression	Makes baculovirus RNA polymerase available for very late gene expression
4	DNA replication and a high concentration of unpackaged viral DNA	High copy numbers of very late genes are accessible for transcription
5	Efficiency of the baculovirus RNA polymerase	Facilitates high level mRNA production and RNA capping
6	The biosynthetic capacity of insect cells	Allows high levels of very late gene expression

Initiating infection: environmental stability and the insect midgut

Insect species are the predominant terrestrial eukaryotic animals both in terms of species numbers and also in their contribution to the terrestrial biomass. After Coleoptera, members of the family Lepidoptera are some of the most numerous in terms of species numbers. Members of this family are phytophagous, and in temperate climates and in many tropical areas where there are distinct wet and dry seasons, insect populations are transitory and expand dramatically during warm, moist periods, and then collapse with the onset of colder temperatures or drought when food sources are reduced or eliminated. In addition, even during seasons optimal for insect growth and reproduction, their populations are normally limited by predators, pathogens, normal temperature cycles, and food sources. However, under certain circumstances, insect populations can expand dramatically when a combination of conditions greatly increases their food supply, facilitates high levels of reproduction, or eliminates predators and pathogens. These cycles of population expansion are well-documented for forest insects and may be separated by long periods of time. The brief life cycle of most Lepidoptera, often limited to a few weeks, allows them to rapidly respond to such environmental conditions. For example, typical lepidopteran larvae, e.g., *M. sexta*, are capable of exponential growth that can be particularly dramatic in the last larval instar, during which a 12-fold increase in mass (from 1g to 12 g) in as few as 4 days has been documented (8). However, considering the small size of their eggs, an overall increase in mass of several thousand-fold occurs over the 3 weeks of their development. The capacity of Lepidoptera to both ingest and assimilate large amounts of food combined with their prolific reproductive abilities are correlated with the devastating losses that they can inflict on both native and cultivated vegetation. One of the purposes of these levels of synthesis is for the production of lipids and proteins that are involved in egg production in females and also in the major transformations that occur during metamorphosis (9). In addition, adult Lepidoptera lack mandibles and either shift to nectar feeding via an extendable proboscis, or do not feed at all, thereby necessitating the storage of energy during the larval stage. Consequently, because of their ability to proliferate to high population densities and their metabolic potential, Lepidoptera provided an attractive target for viral parasitization.

Several major problems had to be solved to allow for baculoviruses to successfully infect Lepidoptera. First, they had to evolve a method of infection. All baculoviruses appear to normally infect insects via the digestive tract as contaminants of the food supply. The insect midgut provided viral access to cells that lacked a resistant chitinous surface. Second, they had to develop a method of persistence so that they could survive the interludes between the population cycles of their hosts. Third, they had to confront the environment of the insect midgut that attains some of the highest pH levels recorded in Nature. At the entry and exit of the midgut, the pH is commonly near 7.0; however, in the central region, levels of 10.0 to as high as 12.0 have been recorded (10) (see Chapter 3, Figure 3). The problems of environmental stability and the high pH of the insect midgut have been solved by several apparently unrelated viruses in a similar manner. This solution involves incorporation of virions into an alkali-soluble occlusion body that provides stability outside the host insect, allowing them to persist in the environment between insect populations. It also provides for the release of the virions when an

environment conducive to infection is encountered. Few if any areas of such high pH are present in nature, except for the lepidopteran midgut. The combination of occlusion with alkali solubility has been so successful that it has apparently evolved independently on at least three occasions: with the baculoviruses, the cypoviruses, and the entomopox viruses. All are insect viruses that infect their hosts via the midgut, but are from completely different viral families. In all cases, the consequence of occlusion is the fact that all these different viruses have developed the ability to express one or two proteins at very high levels. These form the occlusion body matrices.

The infection of insects requires that the occluded virions be liberated from the protein matrix, and this is accomplished by the alkaline conditions encountered in the midgut. Surviving and initiating infection under these conditions is apparently such a highly specialized task that many of the proteins that are involved in this process are conserved throughout not only the Baculoviridae, but also in related groups of nonoccluded viruses, the nudiviruses, and hytrosaviruses. Many of what are considered the more primitive baculoviruses appear to limit their infection to the gut cells. However, when limited to this tissue, their reproductive potential is greatly diminished because they do not have access to systemic infection — in particular, to fat body cells that comprise the major metabolic organ of lepidopteran larvae.

Evolution of a biphasic replication cycle that allowed exploitation of the biosynthetic capacity of insect systems

The ability of baculoviruses to exploit the power of the lepidopteran metabolic system was likely facilitated by their ability to move from the midgut to cause systemic infections. This required the production of a viral type that could initiate infection in a completely different environment: one in which the pH was near neutral and that lacked the high concentrations of enzymes that the occluded virions had evolved to survive. Consequently, baculoviruses appear to have incorporated a more conventional envelope fusion protein, called the F protein, which is similar to the pH-dependent fusion proteins of many viruses. This protein is expressed by the virus, modifies the cytoplasmic membrane and is obtained by the virus when it buds out of the infected cells. It allows the virus to infect a wide variety of cells throughout the insect and thereby provides access to the prodigious metabolic potential of the insect larvae. Subsequently, another fusion protein, GP64, displaced the fusion function of the F protein in one baculovirus lineage (including AcMNPV) and is the focus of much of the research in biotechnology (see Chapter 2).

Optimizing the cellular environment: The viral RNA polymerase and the shut-down of most viral and host genes late in infection

Upon infecting cells, baculoviruses appear to be able to focus transcription on their own genes probably by the expression of the powerful transactivator IE1 along with the presence of enhancer sequences on the viral genome (see Chapter 5). In addition, a major innovation was their incorporation of an RNA polymerase. The viral RNA polymerase allows exploitation of the insect synthetic machinery independent of the constraints of host cell transcription.

In addition to exploiting the synthetic capacity of insects that became accessible by the evolution of systemic infections, several other factors contributed to the evolution of high-level baculovirus gene expression. Identification of one of these factors was based on the observation that most baculovirus genes are shutdown after they have been expressed. An example is the gp64 gene of OpMNPV that has both early and late promoter elements (11). The early promoter is activated shortly after infection (6 hr pi in *L. dispar* cells) and continues until 36 hr after which it declines. Activation of the late promoters occurs between 24 and 36 hr p.i. and then also declines. The vp39 gene, which only has a late promoter element, showed similar kinetics (12). In contrast, the polyhedrin and p10 gene transcripts are present very late in infection (60 hr p.i.) (13, 14). In addition, it has been demonstrated that AcMNPV infections of *S. frugiperda* cells leads to a reduction in levels of almost all host

cell mRNAs by 24 hr p.i. (15). The shutoff of most host genes would likely make both the cellular transcriptional and translational apparatus available so that it can be focused on the expression of viral genes. Similarly, the shutoff of viral late genes likely frees up the viral RNA polymerase so that it can focus on the transcription of polyhedrin and p10. It is not clear what governs the shut off of viral and host genes. The viral transactivator IE1, along with *hr* enhancer sequences, may divert early transcription from host cell genes to viral genes. This could lead to the shut-down of most host genes. The expression of baculovirus late genes occurs after DNA replication is initiated. This could be facilitated by the presence of newly replicated (naked) DNA, or by the presence of the un-ligated junctions of Okazaki fragments that may serve as transient enhancers of late transcription by acting as loading sites for late gene activators that track along the DNA until late promoters are recognized (16) (see Chapter 6). Once DNA replication is completed, these loading sites for late gene activators are no longer available and this may be reflected in the turn off of late genes.

Very late gene (p10 and polyhedrin) activation and transcription

If viral late genes are shut down by the accumulation of DNA binding proteins, very late gene transcription may be induced by the expression of a transcriptional activator (see Chapter 6, Figure 1). As described in Chapter 6, VLF-1 is one of the few genes that has been implicated in the selective up regulation of very late genes and has been shown to interact with the burst sequence of p10 and polyhedrin regulatory regions in gel shift assays (17). LEF-2 (18, 19) and protein kinase 1 (Ac10) (20, 21) (18) (22) may also be involved in this process. In addition to focusing very late transcription on polyhedrin and p10, the high levels of transcription could be influenced by the efficiency of the baculovirus RNA polymerase in transcribing these two genes and also the ability of LEF4 to cap the very late transcripts so that they can be transported and translated efficiently.

A role for gene copy number and non-encapsidated viral DNA

Another major contributing factor to high levels of very late gene expression is likely to be a high copy number of polyhedrin and p10 genes. Baculovirus very late gene expression is fundamentally concerned with the occlusion of nucleocapsids. However, in order for very late genes to be transcribed, it is likely that a significant portion of the DNA synthesized during infection is never packaged into nucleocapsids and is lost at the end of the infection cycle. It is critical that this DNA is not incorporated into nucleocapsids, because once incorporated, it would not be accessible to the RNA polymerase. Indeed, it has been observed that, whereas 100% of BV DNA is resistant to DNAse, up to 70% of viral DNA present in extracts of cells late in infection was sensitive to DNAse, suggesting that it was not packaged and is therefore accessible to digestion (23). The magnitude of gene copy number is reflected in the number of genomes synthesized per cell. AcMNPV and *H. armigera* NPV (HearNPV) were reported to yield up to 81,000 and 131,000 genomes per infected cell, respectively (24) (25). Furthermore, it has been observed that, whereas a portion of the DNA in infected cells can be converted to unit length DNA, most of the DNA appears to be present in complex, possibly branched structures (26). This has led to the suggestion that there are two categories of baculovirus DNA (see Chapter 5): DNA destined to be incorporated into nucleocapsids, and DNA that is never packaged because it is required for very late transcription. The synthesis of DNA incorporated into nucleocapsids may be coordinated with its packaging into virions, whereas the extra-viral DNA is synthesized independent of packaging and undergoes extensive recombination because it is not protected from this process by incorporation into virions. The likely complex structure of the unpackaged DNA would probably not interfere with high levels of very late transcription.

Baculovirus gene expression and biotechnology

As described above, the biosynthetic capacity of baculoviruses is derived from the intersection of their infections with the growth of their hosts. This results in the diversion of the cellular macromolecular material and energetic

capacity to viral rather than insect growth. The ability to recreate this environment in the laboratory was dependent upon the isolation of insect cell lines permissive for baculovirus infection, and the identification of growth conditions that allow for optimal viral replication in these cells. The isolation and development of a variety of insect cell lines to be both permissive for baculovirus replication and to have the capacity to undergo exponential growth has allowed for the exploitation of the combination of both the insect synthetic capacity and the virus in the form of the baculovirus expression system.

Summary and conclusions

It is likely that several factors have combined to make baculoviruses highly efficient in gene expression. This includes gene amplification, the shutoff of most other genes very late in infection, the specific activation of very late genes, efficient gene transcription, and access to the protein synthetic machinery of the host insect, which has evolved to synthesize proteins at high levels to allow the insect larvae to undergo growth and development in a very compact time frame. This is summarized in Table 1.

Whereas equally high levels of gene expression are present in other eukaryotic systems, e.g., the production of ovalbumin in the avian oviduct and the production of various milk proteins in mammals, the expression of these proteins is limited to specific tissues and does not occur systemically. In addition, whereas transgenic animals have been engineered to exploit these systems, they can in no way compare with the ease of manipulating baculoviruses and their cell culture systems for protein production.

Baculovirus Expression Technology: Application

Homologous recombination

The original method for the construction of recombinant baculoviruses was based on homologous recombination and involved the co-transfection of viral DNA along with a plasmid that contained the foreign gene under the control of the polyhedrin promoter and flanked by sequences from the polyhedrin region (1) (2). The selection of recombinants was based on the elimination of the polyhedrin gene and the detection of occlusion negative plaques in cell monolayers. These plaques often occurred at low frequency and their identification required skill and familiarity with the system and often involved extensive screening to confirm that the recombinant expressed the correct insert. This system was improved by a variety of innovations that favored the selection of recombinant viruses, reviewed in (27, 28). One of the protocols involved the linearization of the virus at the polyhedrin locus. Recombination would re-circularize the viral genome and this favored replication (amplification) of the genome (29). Various combinations of this approach were also employed including the incorporation of a lacZ gene at the polyhedrin locus and subsequent linearization, or the further engineering of unique restriction enzyme sites into an essential gene (orf1629) that flanks the polyhedrin locus (30). Linearization using these sites results in a defective genome. The portion of the orf1629 gene deleted in the baculovirus genome was incorporated into the transfer plasmid such that orf1629 function would be restored in recombinants. This along with the elimination of lacZ expression as proof of recombination, greatly facilitated the ability to screen recombinants. However, in recent years the most common approach employs variations of the bacmid system described below.

The bacmid system(31)

The bacmid system exploits two remarkable technologies: the ability of the bacterial transposon Tn7 to specifically integrate adjacent to a defined sequence in bacterial genomes, and the identification of the mini-F plasmid replicon that, when incorporated into the baculovirus genome, allows its coordinated replication in bacteria as an artificial chromosome. The bacmid technology is based on the incorporation of the components of the Tn7 system into a transfer plasmid containing the Tn7 integration signal, and then using a helper plasmid

with transposase functions, and a bacmid that contained the specific Tn7 target sequence within a baculovirus genome, as a targeted method of recombination in bacterial cells. Subsequent isolation of recombinant DNA from bacteria and transfection into insect cells normally results in a viable virus. Because all the added genes except the foreign gene are expressed from bacterial promoters, the genes added to the baculovirus genome are likely to be inactive in insect cells.

The bacmid system: The theoretical basis.

This system exploits a novel transposon called Tn7 that, in contrast to many transposons that insert randomly or into a small target sequence present in many locations, specifically inserts near a highly conserved 36 nt sequence at high frequency.

This system is composed of the transposon Tn7 that was originally identified inserted into a plasmid called R483 because it carried resistance genes to two antibiotics; trimethoprim and streptomycin/spectinomycin) (32). This plasmid was originally isolated from calves that were being fed high doses of trimethoprim (33). In addition to the two resistance genes, Tn7 contains 5 genes that are involved in transposition and flanking sequences of 150-bp (Tn7L) and 90-bp (Tn7R) that are involved in this process (see below). Tn7L contains 3 and Tn7R contains 4 22-bp transposase binding sites. The complete sequence comprises about 14 kb (Figure 1A). In addition to the transposon, the Tn7 system includes a target or attachment site (attTn7) that is the 12 C-terminal codons (36-bp) of the 609-amino acid (in *E. coli*) bacterial protein glmS (34) (35) (Figure 1C). This sequence encodes part of the active site of GlmS, an enzyme involved in the production of N-acetyl-glucosamine, that is a component of an essential cell wall peptidoglycan. Although Tn7 recognizes this sequence, it actually inserts into a sequence located 23 nt downstream of the glmS orf (Figure 1C). Consequently, insertion of Tn7 does not affect the viability of bacteria. In addition, the insertion is normally orientation specific such that Tn7 transcription is in the same orientation as glmS. Although Tn7L and Tn7R show sequence relatedness to each other, they are not related to the AttTn7 sequence (36).

The transposase helper plasmid (Figure 1B)

This construct contains all the genes involved in Tn7 transposition (TnsA-E), a selectable marker TetR, and an origin of replication from the plasmid pBR322. Although it supplies the transposase functions, since it lacks a sequence involved in integration (Tn7L), it will not cause its own integration into other sequences.

Transposition

Transposition is thought to be accomplished as follows: TnsA and TnsB recognize the ends of the transposon (Tn7R and Tn7L), excise it, and then join it to the target DNA. TnsA is related to a category of type II restriction enzymes that cleave DNA downstream of their recognition site in a non-specific sequence– it has been compared structurally to FokI (37), whereas TnsB is related to retroviral integrase. TnsD and E are involved in target selection and activate the transposase machinery that is composed of the TnsA and TnsB. TnsC is an ATPase and is involved in the control of transposition. It interacts with TnsA, activates the TnsAB transposase and is also able to bind to distorted target DNA thereby linking the transposase to the site of insertion.

The baculovirus bacmid

The mini-F replicon

The F (Fertility) plasmid in E. coli K-12 was the first plasmid/bacterial host system to be identified because of its ability to transfer its DNA via a tube-like pilus to bacteria lacking the F plasmid. The F plasmid genome is about 100 kb and encodes about 100 genes that are involved in three main functions i) pilus structure which is essential for cell recognition, contact, and physical transfer of plasmid DNA, ii) conjugative transfer of plasmid DNA, and iii) replication and maintenance of the plasmid in its bacterial host. This latter region was discovered when it was

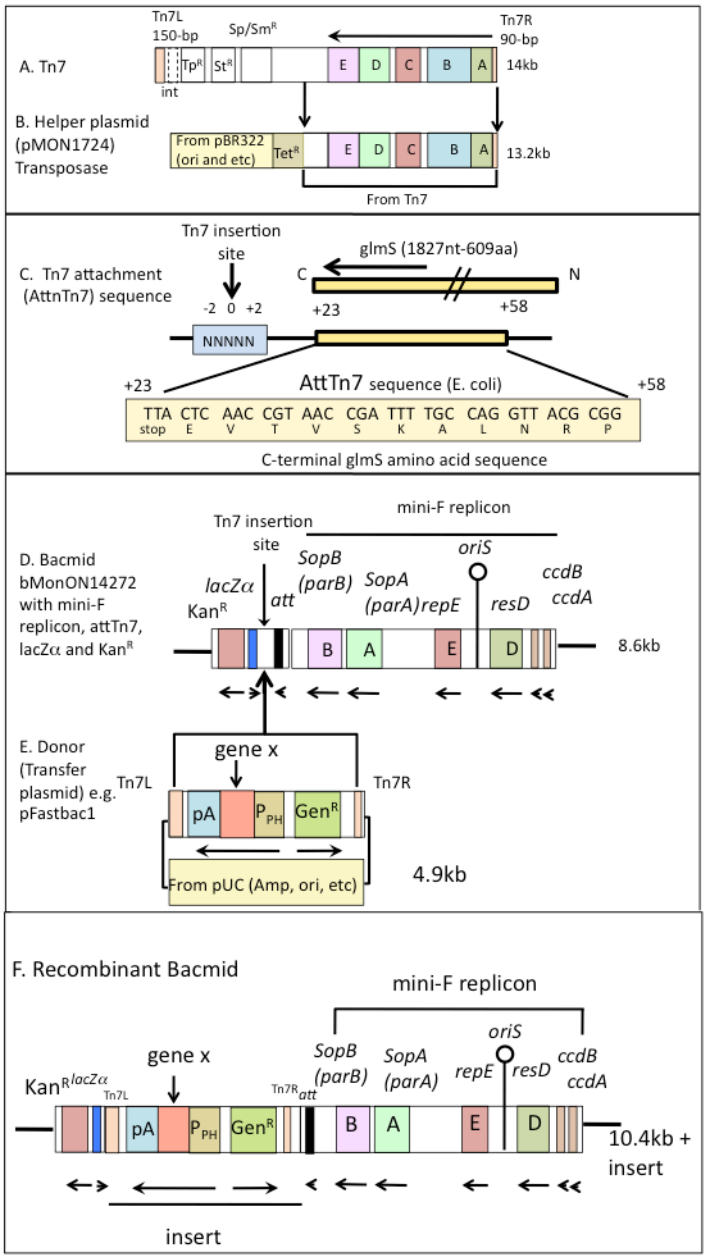

Figure 1. Maps of major constructs used for the production of the bacmid system. A) Map of Tn7. Shown are the genes A-E that are involved in transposition (see text) and at the left end int associated with an inactive integron system and three antibiotic resistance genes including Tp^r (trimethoprim), streptothricin St^r, and streptomycin and spectinomycin Sp/Sm^r (37) (36). B.) Map of the helper plasmid showing the components of Tn7 used in its construction. C) Tn7 attachment sequence. Map of the C-terminal end of the glmS gene showing the Tn7 insertion site 21-25 nt downstream. D). This map shows the organization of the integration site along with the region from the fertility (F) plasmid (mini F-replicon). The genes shown are involved in the coordination of the replication of the bacmid with the bacterial genome (see text). Also shown is the Tn7 integration site (*att*) and the kanamycin resistance gene (Kan^r) and *lacZα*. E.). Map of the donor (transfer) plasmids such as pFastbac1. The location of the polyadenylation signal (pA) polyhedrin promoter (P_{ph}), the selectable marker gentamicin resistance (Gen^R) along with the location of the gene being expressed (gene x). The sequences involved in integration into the att site, Tn7L and Tn7R, are also shown. The rest of the map are the components of the pUC plasmid used for making the transfer plasmid). F.). This is a map of an example of a recombinant bacmid. For abbreviations see above.

found that a 9 kb EcoRI fragment supported replication of a kanamycin resistant plasmid in *E. coli* and was termed the mini-F replicon (38) (39) (40).

Subsequently the mini-F replicon was exploited in the construction of bacterial artificial chromosomes such as bacmids. The incorporation of the mini-F replicon into, for example, a baculovirus genome allows it to replicate

and be stably maintained in bacterial cells such that upon division each daughter cell inherits 1 or 2 copies (41) (42). For the construction of the original baculovirus bacmid, this mini-F replicon sequence was further trimmed to about 7 kb by the elimination of a BamHI-EcoR1 sequence at one end which included an origin of replication, oriV, and two other genes. The remaining sequence contains *oriS,* a unidirectional origin of replication, *repE,* a protein essential for replication that also influences copy number, partitioning (par) proteins *sobA*(*parA)* and *sobB(parB)* that are involved in the segregation of DNA during cell division thereby insuring that daughter cells contain an appropriate DNA complement, *resD* that is required for the resolution of cointegrates, and *ccdA* and *ccdB* (coupled cell division) that are involved in the inhibition of cell division (Figure 1D). The bacmid is further modified by the addition of a Kanamycin resistance gene, the lacZa sequence for color selection, and the Tn7 transposase attachment (att) site (Figure 1D).

The transposon target site

This sequence is from a 550 bp HincII fragment from E.coli glmS C-terminal region and contains the attTn7 sequence (43) (Figure 1C). This is the target sequence where the Tn7 sequences (Tn7L Tn7R) bordering the insert in the transfer plasmid integrate.

The donor (transfer) plasmid (Figure 1E)

The donor plasmid is the cloning vector used for positioning the foreign gene of interest such that it can be transposed into the Tn7 integration site in the bacmid. As an example, pFastBac1 from Invitrogen is shown. The transposable region is bracketed by Tn7R and Tn7L and includes a selectable marker for gentamicin resistance and the 94-bp polyhedrin promoter region which extends from a remnant EcoRV half-site upstream of the ATAAG promoter down to the AT of the polyhedrin ATG. Downstream of the promoter there is a polylinker region that can also include other sequences such as 6xHis tag to facilitate protein purification, a protease sequence allowing subsequent removal of the tag, and by a SV40 polyadenylation signal.

Recombinant bacmid production

The transposase helper plasmid (Figure 1B) is maintained in bacteria by Tet selection along with the bacmid sequence that is maintained by Kan selection (Figure 1D). In the presence of the transfer plasmid (Figure 1E), the transposase causes the insertion of the portion of the transfer plasmid bordered by Tn7L and Tn7R downstream of the att site (Figure 1D) resulting in the recombinant bacmid shown in Figure 1F. Selection is by gentamicin. In addition, the insertion inactivates the production of lacZa, thereby allowing selection of recombinant white colonies using Xgal. Transposition could also occur into the bacterial genome because it encodes the essential glmS gene that contains the attTn7 target sequence, however these colonies should be blue.

Further modification: the Multibac system (44)

The bacmid system has been further modified for the production of multisubunit proteins on a single bacmid. For example, the 1.1 mDa 13 subunit anaphase promoting complex (APC/C) was expressed from two recombinant baculoviruses (8 subunits in one and 5 in the other) (45). In another project the 223 kDa 7 subunit mediator head complex was expressed in a single baculovirus and used for structural determination by X-ray crystallography (46). The multibac vectors use dual very late promoters (polyhedrin and p10) that are inserted into the polyhedrin locus using a system similar to that described above (Figure 1A). It also contains a loxP site in the *v-cath* and *chiA* (Ac126 and Ac127) locus (Ac126 and Ac127 are non-essential in cell culture and are disrupted in this construct). *Vcath* encodes a protease (cathepsin) and elimination of this activity stabilizes expression products, whereas *vchi* encodes a chitinase and its elimination facilitates the application of chitin-affinity chromatography for protein purification. The loxP site allows recombination with a plasmid also containing a loxP site in the presence of cre recombinase that is expressed on a separate plasmid. A set of additional technologies have also been applied. These include the use of internal ribosome entry sites (IRES) to allow translation of tandem protein coding sequences from a single mRNA, the insertion of self-cleaving

polyprotein constructs or by the co-expression of a tobacco etch virus protease (TEVP) and the incorporation of TEVP recognition sequence between the domains of a polyprotein sequence.

Acceptor and donor plasmids

The number of genes incorporated into a transfer vector can be increased using the concept of 'acceptor' and 'donor' plasmids. Acceptor plasmids resemble standard plasmids and contain an origin of replication that allows propagation in *E. coli*. In contrast, the donor plasmids contain a conditional origin of replication that requires an additional protein that is expressed from a modified host *E. coli* strain. Both plasmids contain baculovirus promoters, different antibiotic resistance genes, and loxP sites. Consequently. when the donor and acceptor plasmid DNA along with a plasmid expressing cre recombinase are transfected into the normal E.coli host (that will not support replication of the donor plasmid) and subjected to selection with both antibiotics, the surviving plasmid will be a recombinant expressing both antibiotic genes. The plasmids also contain unique endonuclease sites, e.g. BstXI or homing endonuclease sites that can facilitate the insertion of additional genes. Homing endonucleases are encoded by introns or inteins and have long recognition sequences. The insert region is flanked by the Tn7L/Tn7R sequences and once the desired set of genes is assembled, it can be inserted into the bacmid sequence at the *att* site using the Tn7 transposase system (Figure 1). Further genes can be incorporated into donor plasmids and recombined into the *v-cath/chiA* locus. For construction of the APC/C 8-subunit complex, a plasmid containing 6 genes was inserted using the Tn7 system, whereas the other two genes were located on another plasmid and inserted into the *v-cath/chiA* locus using *cre* recombinase. The other bacmid was made by incorporating 5 genes into the Tn7 *att* locus (45).

The flashbac system. A hybrid bacmid-homologous recombination system (28)

This novel technology for the production of recombinant baculoviruses combines the advantages of bacmid technology with homologous recombination in insect cells. A defective baculovirus/bacmid construct is used that replicates in bacteria, but when co-transfected into insect cells with the transfer vector, the only viable recombinants are ones that are derived from a plasmid that repairs the defect in the baculovirus genome. To accomplish this, a bacmid was engineered that lacks a portion of the C-terminal region of orf1629 (Ac9) that is adjacent to the polyhedrin locus. Orf1629 is an essential gene and encodes a structural protein located at the base of the virion that is involved in actin assembly (see Chapter 3). Orf1629 is repaired during the recombination event and results in a viable virus with the gene of interest inserted into the polyhedrin locus. This approach has a number of advantages over other baculovirus expression systems including the elimination of the need for i) antibiotic or lacZ screening, ii) the transposase encoding helper plasmid, and iii) plaque purification. In the process of recombination, the mini-F replicon region is eliminated since it is flanked by the lef2 (ac6) and orf1629 genes that border the polyhedrin gene locus and are the sites of recombination (Figure 2).

In the original *flash*BAC construct, the chitinase (*chiA*) (Ac126) gene was deleted because it was observed to interfere with the secretory pathway in the endoplasmic reticulum. Deleting *chiA* improved the expression of secreted and membrane proteins. In a subsequent vector, *flash*BACGOLD, the baculovirus cathepsin (*vcath*) (Ac127) gene that encodes a proteinase was deleted in addition to *chiA*. This reduces the proteolysis of expressed proteins. A subsequent vector, *flash*BACULTRA has an additional three adjacent genes deleted, p26 (Ac136), p10 (Ac137), and p74 (Ac 138). Since p10 is a highly expressed very late gene, its elimination reduces competition with the polyhedrin very late promoter, p74 is required for infection of insect midgut cells and its removal prevents insect transmission by the recombinant virus. The mutation does not affect replication in cultured cells. P26 is not well characterized, but its deletion appears to have no effects in cell culture.

These constructs are also available as a BacMagic series from EMD Millipore, Inc., a division of Merck, KGa.

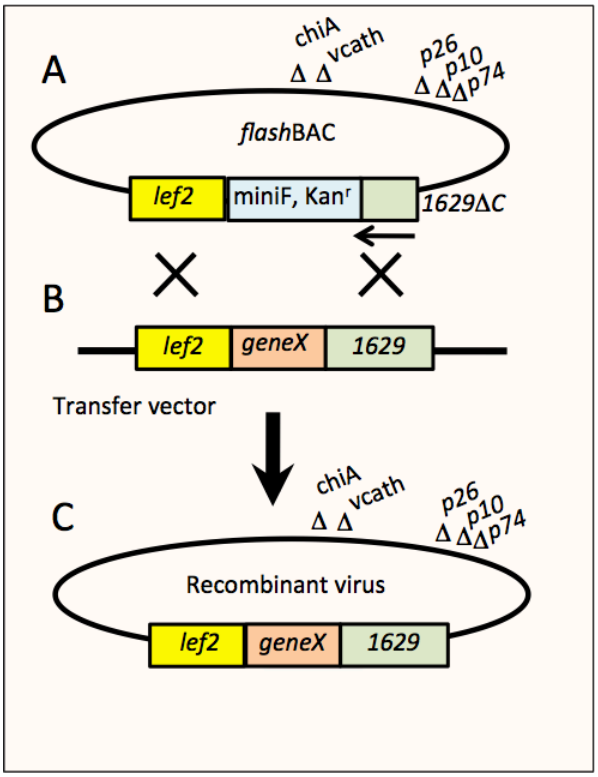

Figure 2. The *flash*BAC baculovirus expression system. A.). A map of the *flash*BAC bacmid. The deleted genes are indicated. The mini-F insert contains genes from the F plasmid involved in coordination of replication of the bacmid with the bacterial genome (see Fig. 1D). A kanamycin resistance gene is also indicated. The essential Orf 1629ΔC is truncated at its C- terminus. This prevents the bacmid from replicating as a virus in insect cells. B). The transfer vector which contains a flanking gene, lef2, the insert, and a complete orf1629 gene. C.). A recombinant virus with a repaired orf1629 gene. The BAC sequences are eliminated by recombination.

OmniBac

OmniBac is a system allowing either transposon mediated recombination or homologous recombination based on the orf1629/lef2/orf603 sequences for insertion of multiple genes into baculovirus vectors. It allows the use of all baculoviruses using either of these two approaches (47).

Post translational processing of baculovirus expressed proteins

Glycosylation

The baculovirus expression system has been widely used because of its ability to process proteins similar to other higher eukaryotes. This leads to proteins that are often biologically active as a result of being folded properly, trafficked to their native locations in the cell, and posttranslationally modified similar to higher eukaryotic cells. One of the areas of difference between the insect system and that of higher eukaryotes is in glycosylation. Whereas higher eukaryotes such as mammals have complex N-glycans with sialic acid residues at their termini, those produced in the baculovirus system have a simpler side chain terminating in mannose residues with the addition of 1-3 fucose residues. Although it has been reported that some insect cell lines are capable of the synthesis of more complex sialated glycans, the proteins examined in these reports may have been contaminated with a sialoglycoprotein in the culture medium (48). The fucose residues found in insect derived glycoproteins are of concern because they can elicit a strong allergic response in some humans due to the presence of cross-reactive IgE derived from environmental exposure to the fucose epitope; reviewed in (49).

In both insects and mammals, the initial N-glycan complex is added to asparagine residues (in the context of Asn-X-Ser/Thr) in the endoplasmic reticulum or Golgi apparatus. This normally involves the addition of a pre-assembled precursor glycan comprised of two N-acetyl-glucoseamine residues attached to a branched complex of mannose molecules with one branch further extended by the addition of glucose molecules (Figure 3A). This complex is then processed by the addition or removal of different carbohydrate molecules. Two main reactions are involved; glycosidases and mannosidases remove (50) glucose and mannose molecules, respectively (Figure 3B), whereas a glycosylaminotransferase is involved in the addition of a GlcNac residue (Figure 3C). Although the initial N-glycosylation structure added to the protein and the preliminary trimming reactions are similar between insects and mammalian cells, the succeeding processing reactions differ significantly.

In the insect pathway, the action of fucosyltransferases and an N-acetyglucosamidase, result in one non-fucosylated and two different fucosylated variants (Figure 3E). In contrast the mammalian pathway can yield several sialated and non-sialated variants (Figure 3F).

Because of the possibility of the fucosylated proteins eliciting an allergic reaction, efforts has been made to identify the enzymes involved in the production of glycan modifications and incorporate them into insect cells so that N-glycans that resemble those found in mammalian cells are produced; reviewed in (51). This has resulted a commercially available transgenic *Spodoptera frugiperda* cell line (SfWT-series) (Mimic cells, Invitrogen, Inc.) capable of producing terminally sialylated N-glycans similar to those found in mammalian systems (52) (53).

A recent system called Sweetbac (54) incorporates genes coding for bovine β1,4-galactosyltransferase I and Caenorhabditis elegans N-acetylglucosaminyltransferase II into a multibac vector (see above) such that proteins expressed by this virus had a mammalian-like N-glycan structures.

Another innovation involves the problem of sialylation which involves the added expense of media supplementation with a precursor of sialic acid, N-acetylmannosamine. To eliminate the need for this supplement, the gene encoding *E. coli* N-acetylglucosamine-6-phosphate 2'-epimerase (GNPE) was engineered into insect cells. It was found that, although GNPE normally degrades sialic acid, in insect cells which have the degradation product, but not sialic acid, the reverse reaction (sialic acid synthesis) occurs (55).

Further baculovirus manipulation: Synthetic baculovirus technology

Using PCR and transformation-associated recombination in yeast, a viable synthetic AcMNPV genome was constructed. This technology may facilitate the investigation of gene function which could be important in advances in gene expression technology and basic biology (56).

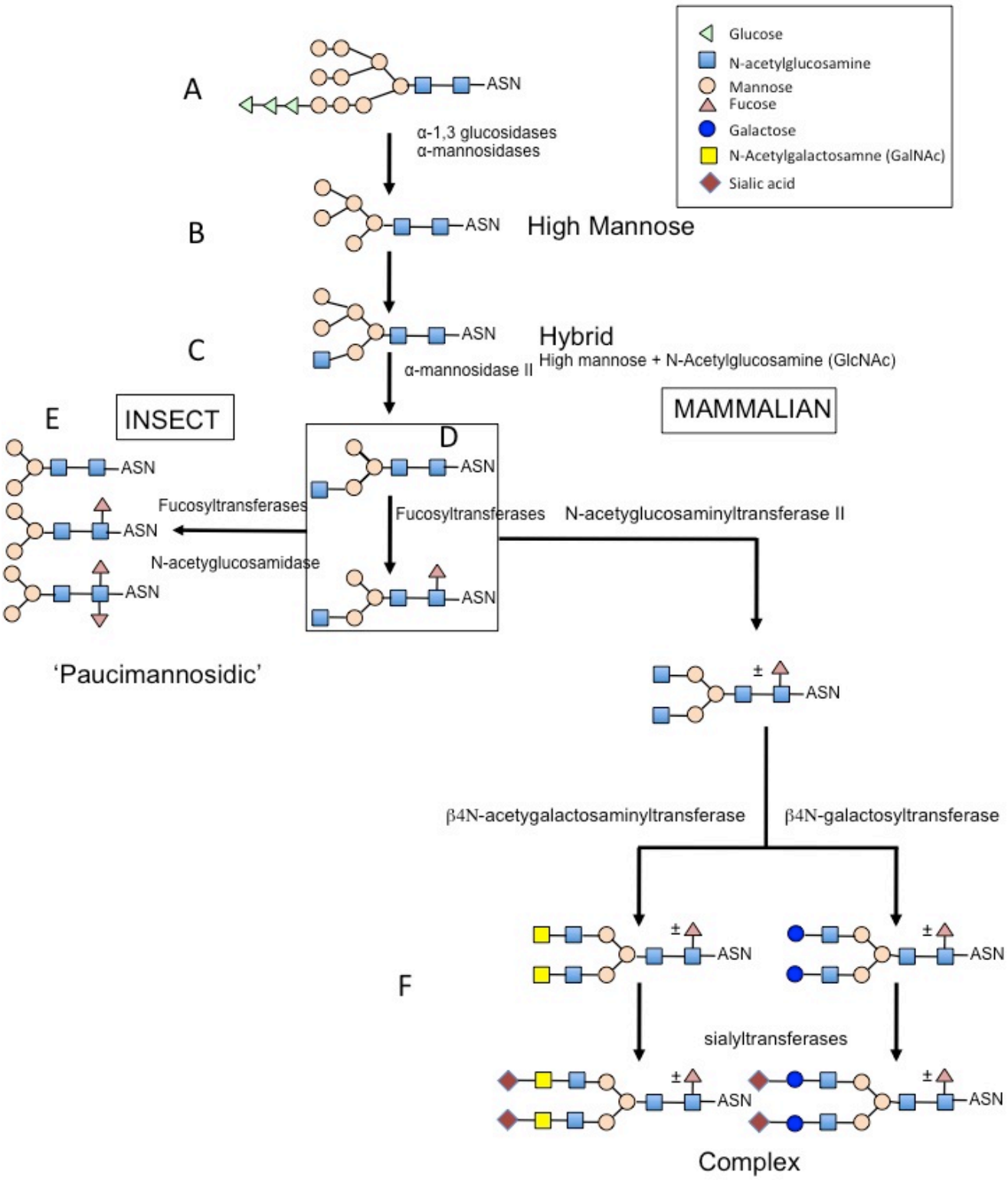

Figure 3. A comparison of insect and mammalian N-glycosylation pathways. The symbols are defined in the key. The major difference is that insect derivatives may contain fucose (D), whereas the mammalian derivatives may or may not incorporate fucose (E) and contain N-acetylgalactosamine (GalNac) or with or without sialic acid (F). Modified from (51).

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