Developing insect gene expression system in the genome era

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1. Introduction

Among a wide variety of gene expression systems using both prokaryotic and eukaryotic cells, the baculovirus expression vector (BEV) systems, especially the *Autographa californica* nucleopolyhedrovirus (AcNPV) vector system, are now frequently used in many fields of both basic and applied biology as a convenient tool. In BEV systems, strong viral very late promoters, such as the polyhedrin and p10 promoters, enable the high-level expression of heterologous genes in insect cells and the resulting products are usually correctly folded and modified as eukaryotic fashions so that possess structures and biological activities similar to the native counterparts. In spite of these advantageous characteristics, several limits of BEV systems, such as adverse effects on the host cell function by lytic virus infection and insect cell-specific properties of posttranslational modification, hamper the practical large-scale production of pharmaceutical proteins.

Until recent, most improvements of BEV systems have been made by engineering the virus genome DNA and/or searching for alternative virus/host cell (insect) combinations showing better performance in the recombinant protein production. These strategies obviously just optimize production efficiencies within the capacity of intact host cell whose precise mechanisms are unknown. In fact, we have developed two BEV systems using *Hyphantria cunea* NPV (HycuNPV) (Takenaka *et al.*, 1999; Shinoda *et al.* 2001) and *Antheraea pernyi* NPV (AnpeNPV) (Wang *et al.*, 2000; Kobayashi *et al.*, 2001; Huang *et al.*, 2001), and found it not so difficult to establish novel BEV systems with productivities comparable to those of existing systems, but difficult to make them superior. However, advantageous characteristics of the AnpeNPV vector system indicated us ideal models of BEV system, which can be realized by controlling expression of certain sets of genes in the host cell. Fortunately, the recent progress in the insect genome projects has begun to uncover insect cell functions as gene networks. Thus, insect cells are becoming the real targets of thorough manipulation by metabolic engineering and no longer black boxes to be analyzed. The growing knowledge on both baculovirus and host insect genomes will allow us to develop novel methodologies based on molecular mechanisms of virus and host cell interactions for utilizing insect cells as excellent protein production factories in this insect genome era.

2. Construction of AnpeNPV vector system

There are two possible ways for the production of recombinant proteins in BEV systems, one of them is *in vitro* production using insect cell lines and the other is *in vivo* production using insect larvae. In general, recombinant proteins produced *in vitro* are relatively homogeneous and can be easily purified from culture medium and/or infected cells. However, costs of media and equipments for the cell culture are very expensive for the economical scale-up. In contrast, large amounts of recombinant proteins can be produced at lower costs by the *in vivo* system using *Bombyx mori* larvae which have been completely domesticated and whose rearing technology has been quite sophisticated in the long history of the sericulture (Maeda, 1989). In addition, the tissue-specific and complicated post-translational modifications, which are not observed in cultured insect cells, can be occurred in various differentiated cells in

larvae, although resulting recombinant proteins become more heterogeneous than *in vitro* production. The major drawback of *in vivo* production is difficulty in the purification of recombinant proteins from large amounts of larval proteins as well as other materials. *In vivo* production also requires laborious tasks of larval manipulation, such as recombinant virus inoculation and hemolymph and/or tissue collection.

For *in vitro* production system using the AcNPV, many technical improvements have been added and several user-friendly kits are now available from various companies. A few kits of *in vivo* production using *B. mori* larvae are also available, however, it is very difficult for any laboratories without insect rearing facility to introduce these kits. To avoid this inconvenience, diapausing pupae, which can be purchased and maintained for a long period in refrigerator without any foods until using for the virus inoculation, are considered as an ideal alternative to larvae. *B. mori* does not diapause at pupal stage but at early embryonic stage. Therefore, diapausing pupae of giant silk moths such as *Hyalophora cecropia* and *Antheraea pernyi*, both of which belong to the genus Satruniidae, were chosen in the pioneered studies (Hellers and Steiner, 1992; Zhang *et al.*, 1992). After publications, these BEV systems never became widely used, even though the high level expression of heterologous genes was demonstrated.

Recently an *A. pernyi* cell line, NISES-AnPe-428 (AnPe), was established (Inoue and Hayasaka, 1995). We have demonstrated that AnPe cells support replication and plaque-purification of the wild type AnpeNPV (Wang *et al.*, 2000) and succeeded to construct a transfer vector plasmid pApCH1, which enable to express foreign genes under the strong polyhedrin promoter of AnpeNPV (Kobayashi *et al.*, 2001). In order to evaluate the efficiency of the protein production in the AnpeNPV vector system, we have compared the *E. coli lacZ* gene expression among several BEV systems (Huang *et al.*, 2001).

As shown in Fig. 1, β-galactosidase activity by the *in vitro* production using AnPe cells was higher than BmN4 cells, comparable with Sf9 and SpIm cells and lower than High5 cells. The speeds of production in the AnPe cell culture were slower than the other *in vitro* BEV systems. In the *in vivo* production using *A. pernyi* pupae, β-galactosidase activity per gram insect in female pupae was about 1.3-fold higher than that in male pupae. The fact that the female pupa (about 12 g) is 1.5-fold heavier than the male pupa (about 8 g) means that one female pupa can produce equivalent quantity of recombinant protein with that produced by two male pupae. *B. mori* 5th instar larvae showed similar productivity to *A. pernyi* male pupae and less than female pupae. The speeds of production in *A. pernyi* pupae were much slower than in *B. mori* larvae. Thus, the slow production of recombinant protein seemed to be an identical characteristic of AnpeNPV vector system, probably reflecting moderate speeds of AnpeNPV multiplication in *A. pernyi* cells. In addition, the virus multiplication may be further delayed under physiological conditions in diapausing pupae, such as slow diffusion of free virions in pupal body fluids and retarded viral DNA replication in diapausing cells.

An obvious drawback of the slow production in diapausing pupae was significant degradation of recombinant protein at later stage of infection. However, similar proteolytic degradation of recombinant protein was commonly, although less than in *A. pernyi* pupae, observed not only in *B. mori* larvae but also more or less in all the insect cell cultures. It is well known that cysteine protease (cathepsin) gene (*v-cath*) is encoded in the baculovirus genome and expressed at late stage of infection (Ohkawa *et al*, 1994, Hawtin *et al.*, 1997). Thus, it is likely that the degradation of β-galactosidase in *A. pernyi* pupae had become so remarkable because of longer incubation time with cathepsin. In the BmNPV vector system, proteolytic degradation of recombinant protein was virtually suppressed by deleting the *v-cath* coding region from the viral DNA genome (Suzuki *et al.*, 1997). By comparing genome DNA maps of AnpeNPV and OpMNPV (Ahrens *et al.*, 1997), we have already identified the AnpeNPV *v-cath* homolog and constructed similar protease-free AnpeNPVs. In addition, we

had a preliminary result that β-galactosidase production per gram insect increased more than twice when diapausing pupae of *Samia cynthia pryeri*, another satruniid moth, were used as alternative hosts for AnpeNPV infection (Fig. 1). Altogether, our results suggested that the AnpeNPV vector system using diapausing pupae of wild silkmoths has a potential to become more practical protein production system than *B. mori* larvae.

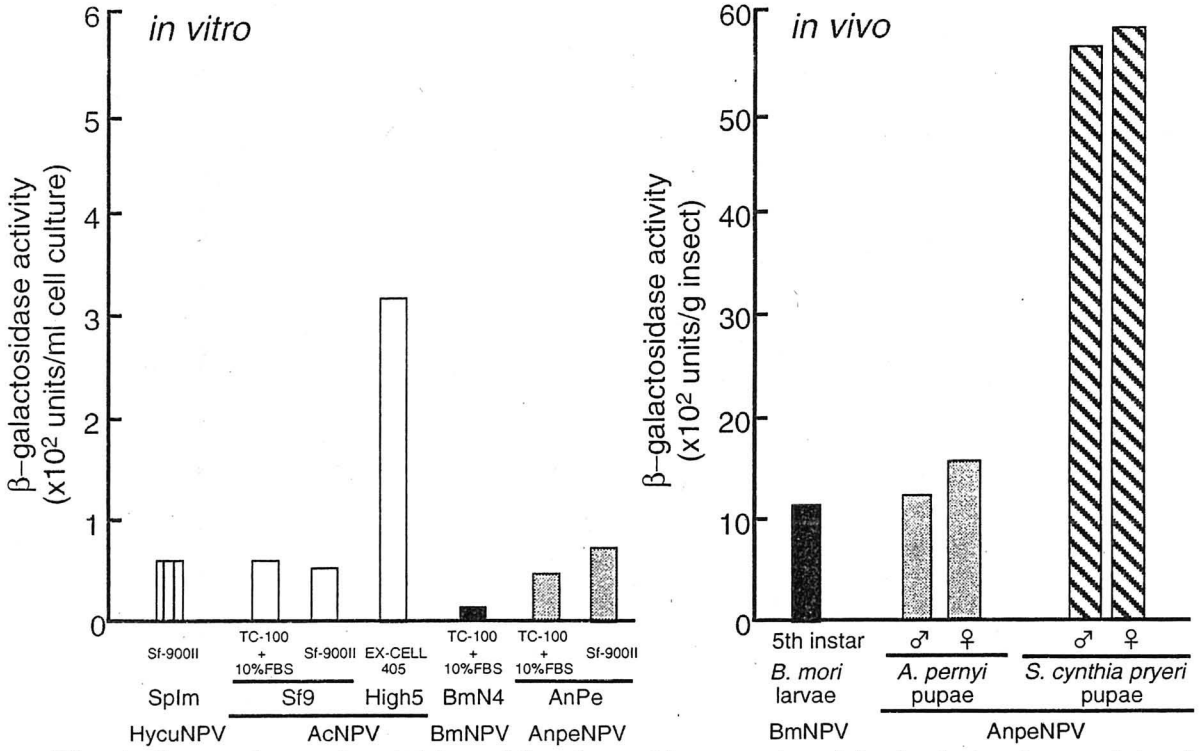


Fig. 1. Comparison of activities of β-galactosidase produced by both *in vitro* and *in vivo* baculovirus expression vector systems

3. Metabolic engineering of N-glycosylation pathway

N-glycosylation is a well studied and characterized posttlanslational modification process of lepidopteran insect cells used in BEV systems. In general, truncated oligosaccharides containing two or three mannose residues and often one or two fucose residues are attached to recombinant glycoproteins produced in BEV systems (Fig. 2). It has been demonstrated that a recombinant human glycoprotein produced in BEV systems is cleared more rapidly from the mammalian circulatory system, probably because of the structure of insect-cell derived N-glycans. In addition, core α-1,3-fucose found in parts of N-linked oligosaccharide side chains added by insect cells may act as a strong allergen (Altmann *et al.*, 1999). Previous works on searching for the alternative host cell lines with capability to produce complex type N-glycans observed in mammalian cells have revealed variations in N-glycosylation property among cell lines and a few examples of N-linked side chains with terminal galactose or sialic acid added on glycoproteins produced by the BEV systems, although these complex type N-glycans were only minor components and were not always detectable, resulting in inability to use them for further practical use (Jarvis, 1997). We have also observed positive bands indicating addition of terminal galactose, and possibly terminal sialic acid, to N-glycan on the recombinant prothoracicotoropic hormone (PTTH) produced by AnPe cells in the lectin blot analysis (Fig. 3). However, the detected bands were faint and less reproducible. Further characterization of N-glycans on PTTH produced by AnpeNPV vector is now in progress. Thus, the complex N-

glycan formation in the AnpeNPV vector system is still controversial as similar results reported for other BEV systems (Jarvis, 1997).

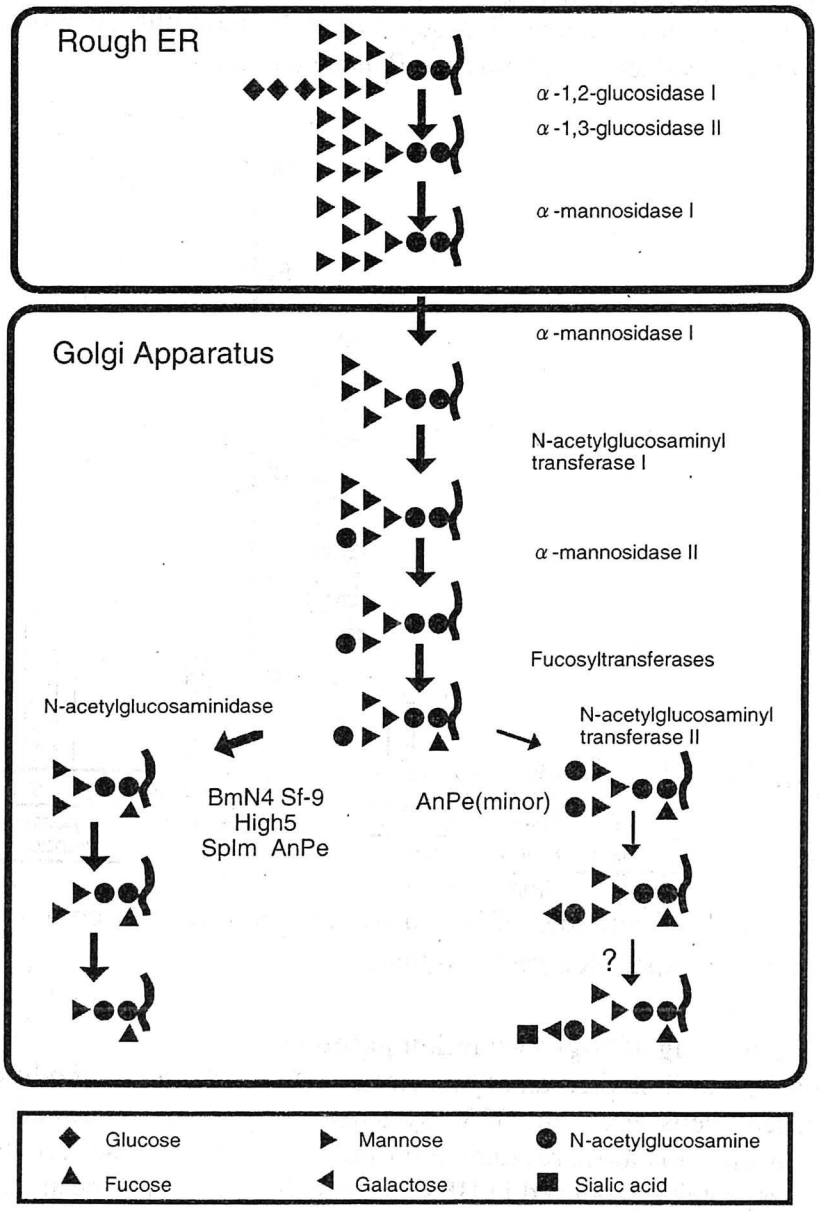
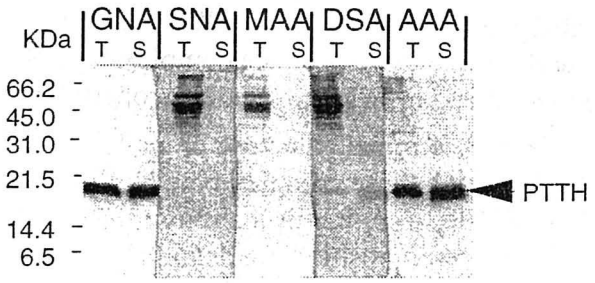


Fig. 2. Estimated pathways of the N-glycosylation in various insect cell lines



Lectin	Sugar
GNA	Man α (1-3) Man
SNA	NeuNAc α (2-6) Gal/GalNAc
MAA	NeuNAc α (2-3) Gal
DSA	Gal β (1-4) GlcNAc
AAA	Fuc α(1-6) GlcNAc

T : TC-100(10%FBS)
S : Sf-900II

Fig. 3. Lectin blot analysis of PTTH expressed in AnPe cells

Such an ambiguous situation will not continue so long. Sooner or later, most of the genes involved in the N-glycosylation pathway of some model insects, particularly *Drosophila melanogaster*, will be identified and then the homologs of these genes in lepidopteran insects will be identified. In fact, several candidate genes for the complex N-glycan formation, such as N-acetylglucosaminyltransferase I, β-1,4-galactosyltransferase and α-2,6-sialyltransferase gene homologs, along with those of impeditive or unnecessary genes, such as N-acetylglucosaminidase and a-1,3-fucosyltransferase genes, have already found in the *Drosophila* genome sequence. No homologs of these *Drosophila* genes except N-acetylglucosaminidase gene have found in the EST database of *B. mori* (Silkbase), probably because the Silkbase does not cover the whole *B. mori* transcripts.

We, therefore, decide to use *Drosophila* S2 cells for a time to establish methods for metabolic engineering of insect N-glycosylation pathway by primarily enhance and/or depress the expression of certain sets of endogenous genes to enable the production of safe therapeutic glycoproteins with complex N-glycans in insect cells. It has recently reported that terminal galactose and/or sialic acids were certainly added to N-linked oligosaccharides on glycoproteins produced in Sf9 cells by introducing one or two mammlian genes responsible for the complex N-glycan formation, although only a minor part of the N-glycans was converted to the complex-type (Jarvis *et al.*, 2001). In contrast, our metabolic engineering strategy has a potential to increase largely and stably the population of complex N-glycans, if the whole process of insect N-glycosylation pathway is adequately modified after a continuing process of trial and error.

4. Conclusion - Toward creation of diapausing insect cell culture as an advanced insect factory

Even for *Drosophila*, whose whole genome sequence had already determined (Adams *et al*, 2000), its precise cellular mechanisms at molecular level are still beyond our grasp. Much more knowledge and new methods are required for accomplish the fundamental alteration of N-glycosylation pathway. For lepidopterans, to make matters worse, only a few genome projects smaller than the *Drosophila* genome project are now in progress in the world. I expect steady and intensive growth of these projects, especially for the silkworm genome project in Japan, because without abundant data from these projects it is very difficult or even impossible not only to transfer techniques for metabolic engineering of N-glycosylation pathway from *Drosophila* S2 cells to lepidopteran cells but also to create a novel BEV system utilizing genetic mechanism involved in insect diapause. As demonstrated by AnpeNPV vector system, diapausing pupa is an excellent natural bioreactor for the protein production with a long shelf life under conditions without any nutritional inputs. If a kind of insect stem cells which can get into and out of diapausing state with complete control is established, in vitro bioreactors with properties much better than pupa will be constructed by combining diapausing insect cell culture and metabolic engineering technology.

The diapausing insect cell culture is yet a concept and may sound like a pie in the sky , but I do believe that it will be an ideal BEV system of worth creating in this insect genome era.

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