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Improvement of Baculovirus Expression Vector System Based on Genome Sequence of *Antheraea pernyi* Nucleopolyhedrovirus

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Abstract **The nucleopolyhedrovirus of Chinese oak silkmoth, *Antheraea pernyi*, (AnpeNPV) isolated in Liaoning Province, China, was plaque-purified by using the cell line NISES-AnPe-428 derived from *A. pernyi* embryos. We employed one of the purified virus, clone A, for constructing a baculovirus expression vector system and succeeded the efficient recombinant protein production in diapusing pupa which is an excellent natural bioreactor storable more than one year in refrigerator. To understand molecular mechanisms of the virus - host cell interaction and improve the vector system, we have sequenced the circular dsDNA genome of AnpeNPV clone A and revealed that the genome is 126,592 bp in size, relatively GC-rich (53.4% G+C), with 150 predicted open reading frames (ORFs), 95% of which (142 ORFs) are homologues in other baculoviruses. Based on the absence of *Avr*II restriction site in the genome, two *Avr*II sites have been introduced at 5' and 3' UTRs of the polyhedrin gene for linearizing the AnpeNPV DNA. In addition, attR1 and attR2 sites for site-specific cloning of the gene of interest from Gateway® (Invitrogen) entry clone have been inserted just upstream and downstream of the two *Avr*II sites, respectively. The resulting viral DNA has allowed us to obtain recombinant virus by direct gene transfer *in vitro* without any purification steps, demonstrating the virus vector applicable for high-throughput production of proteins.**
Key words: Nucleopolyhedrovirus, *Antheraea pernyi*, genome, baculovirus expression vector system

In 1990, the First International Conference on Wild Silkmoths was held in Shenyang, China, and our collaborative study on the development of baculovirus expression vector (BEV) system using *Antheraea pernyi* nucleopolyhedrovirus (AnpeNPV) had started (Fig. 1).

One year later, a cell line forming contractile muscle-like networks *in vitro* was obtained from *A. pernyi* embryos (Inoue *et al.*, 1991). From the cell line, non-contractile cells with good proliferation

were isolated and designated as NISES-AnPe-428 (AnPe) (Inoue and Hayasaka, 1995). The AnPe cells had proved to be susceptible to infection of the AnpeNPV isolated in Liaoning Province, China, and used for plaque-purification of the virus, resulting in three virus clones (A, B and C) (Wang *et al.*, 2000) (Fig. 2).

Then we constructed the baculovirus expression vector (BEV) system using the clone A of AnpeNPV (Kobayashi *et al.*, 2001) and evaluated its performance in protein production and post-translational modifications, especially *N*-glycosylation, as a part of the center of excellence (COE) project "Research for Utilization of Insect Properties" by the National Institute of Sericultural and Entomological Science from 1996 to 2000.

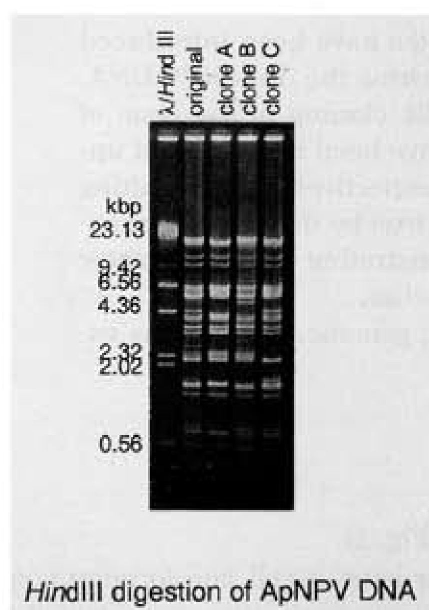
- 1990 1st International Conference of Wild Silkmoths
- 1991 *A. pernyi* cell line AnPe
- 1994 AnpeNPV clone A
- 1996 ~ 2000 COE project
- 2001 Baculovirus expression vector system
- 2002 AnpeNPV genome map
- 2006 BaculoTech Co., Ltd.
- 2007 Complete AnpeNPV genome sequence
- 2009 Gateway adapted AnpeNPV vector system

Fig. 1. Short history of the AnpeNPV BEV system development.

When the recombinant protein (β-galactosidase) production efficiency of AnpeNPV BEV system using AnPe cells and diapausing pupae of wild silkmoths such as *A. pernyi* and *Samia cynthia pryeri* was compared with those of other BEV systems such as *Autographa californica* NPV with Sf9 and High5 cells, *Bombyx mori* NPV with BmN4 cells and *B. mori* larvae, and *Hyphantria cunea* NPV with SpIm cells, the AnpeNPV-infected diapausing puape of *S. c. pryeri* showed the highest protein production efficiency (Huang *et al.*, 2001; Kobayashi, 2001) (Fig. 3).

Thus, The most attractive property of the AnpeNPV BEV system is that any insect rearing facilities are not required for the recombinant protein production using diapausing pupae of wild silkmoths. Users just have to purchase the pupae and store in refrigerator until injecting recombinant viruses.

In addition, it has been found that the structure of some *N*-glycans added to the recombinant glycoprotein, prothoracicotropic hormone (PTTH), by AnPe cells is bianntenary complex type which is not detected in Sf9 cells but typical in mammalian cells, suggesting that AnPe is well suited for producing



- Virus
  Antheraea pernyi NPV (AnpeNPV)
  clone A (from China, 1994)
- Cell line
  NISES-AnPe-428 (AnPe)
  from embryos of *A. pernyi*
  (Inoue & Hayasakai, 1995)
- Medium
  TC-100 or Grace (with 10%FBS) medium
  Sf-900II or Cosmedium 009 serum free medium
- Insect
  Diapausing pupae of *A. pernyi*
  and other satruniid moths

Fig. 2. Electrophoretic profiles of *Hin*dIII-digested DNA genomes of three AnpeNPV clones (left) and outline of AnpeNPV BEV system (right).

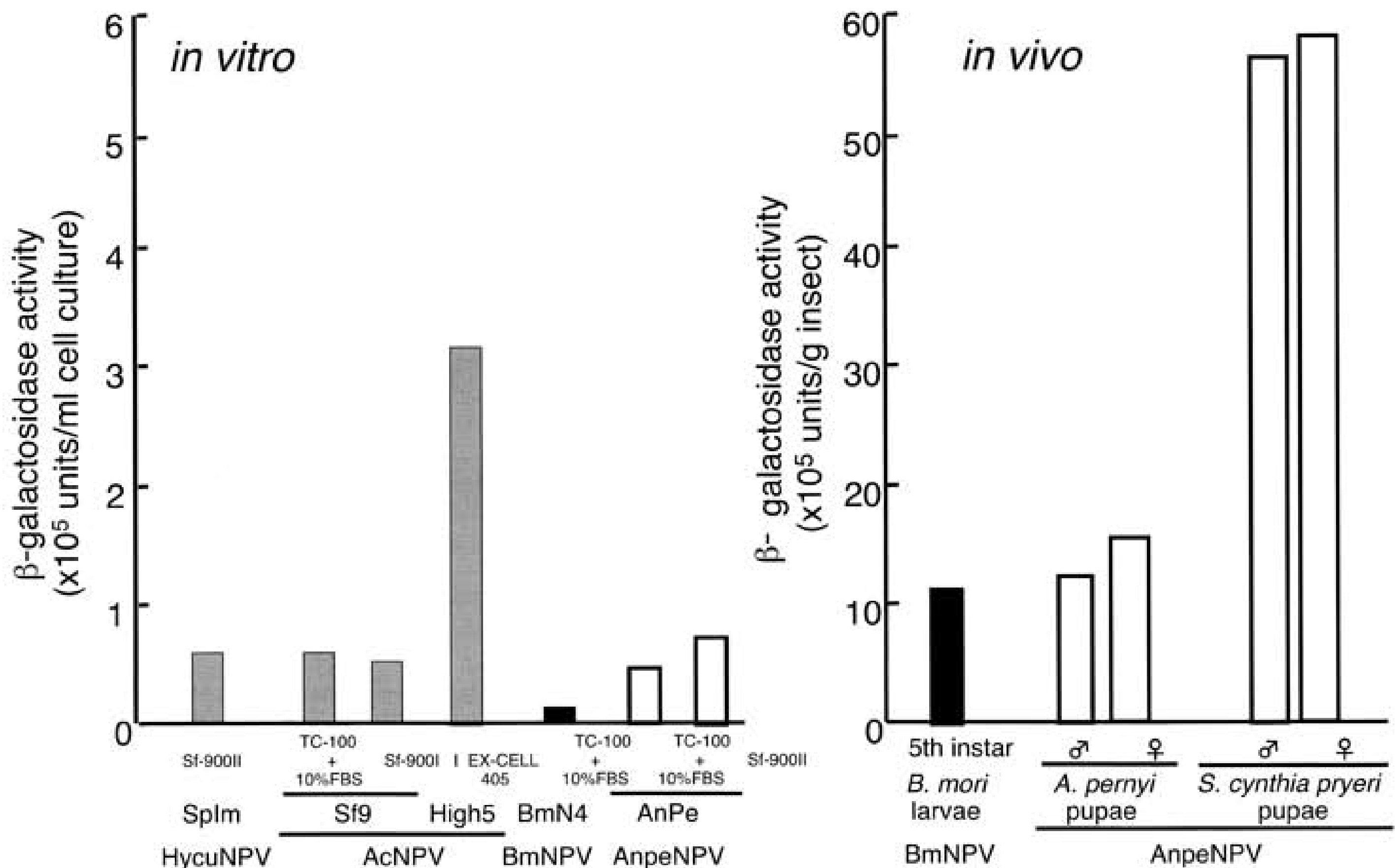


Fig. 3. Evaluation of recombinant protein production efficiency of AnpeNPV BEV system by comparing β-galactosidase activities among several BEV systems.

pharmaceutical glycoproteins with mammlian-like *N*-glycans (Nagaya *et al.*, 2002, 2003) (Fig. 4). In 2006, we had founded a venture company, BACULOTECHNOLOGIES CO., LTD. and started the recombinant protein production service using the AnpeNPV BEV system.

A comparative genome map of AnpeNPV was constructed by aligning 54 *Pst*I fragments with the fully sequenced *Orgyia pseudotsugata* multicapsid NPV (OpMNPV) genome (Ahrens *et al.*, 1997) (Fig. 5) and used for identifying and cloning several virus genes to improve protein production capabilities as well as to expand host range by targeting or modifying them. (Huang *et al.*, 2002; Huang and Kobayashi, 2003; Maegawa *et al.*, 2003; 2004).

For further improvements of the AnpeNPV BEV system by investigating and manipulating virus genes, we have sequenced the 54 *Pst*I fragments (Huang *et al.*, 2007). According to the assembled sequence, the genome is 126,592 bp in size, relatively GC-rich (53.4% G+C), with 150 predicted open reading frames (ORFs) of more than 48 amino acids. 95% of the ORFs (142 ORFs) have predicted functions or homologues in other baculoviruses, and their homology search results using BLAST program as well as their organization in the genome have clearly indicated that the AnpeNPV is a member of Group I NPV and closely related to *Orgyia pseudotsugata* MNPV and *Hyphantria cunea* NPV (Ikeda *et al.*, 2006). Although the genome sequence of clone A is very similar to those of two other Liaoning isolates of AnpeNPV (GenBank accession numbers, NC_008035 and EF207986) independently determined by Chinese research groups (Nie *et al.*, 2007; Fan *et al.*, 2007), detailed characteristics such as genome size, number of ORFs, length of each ORF are varied each other, suggesting the considerable genome sequence diversity in the natural AnpeNPV

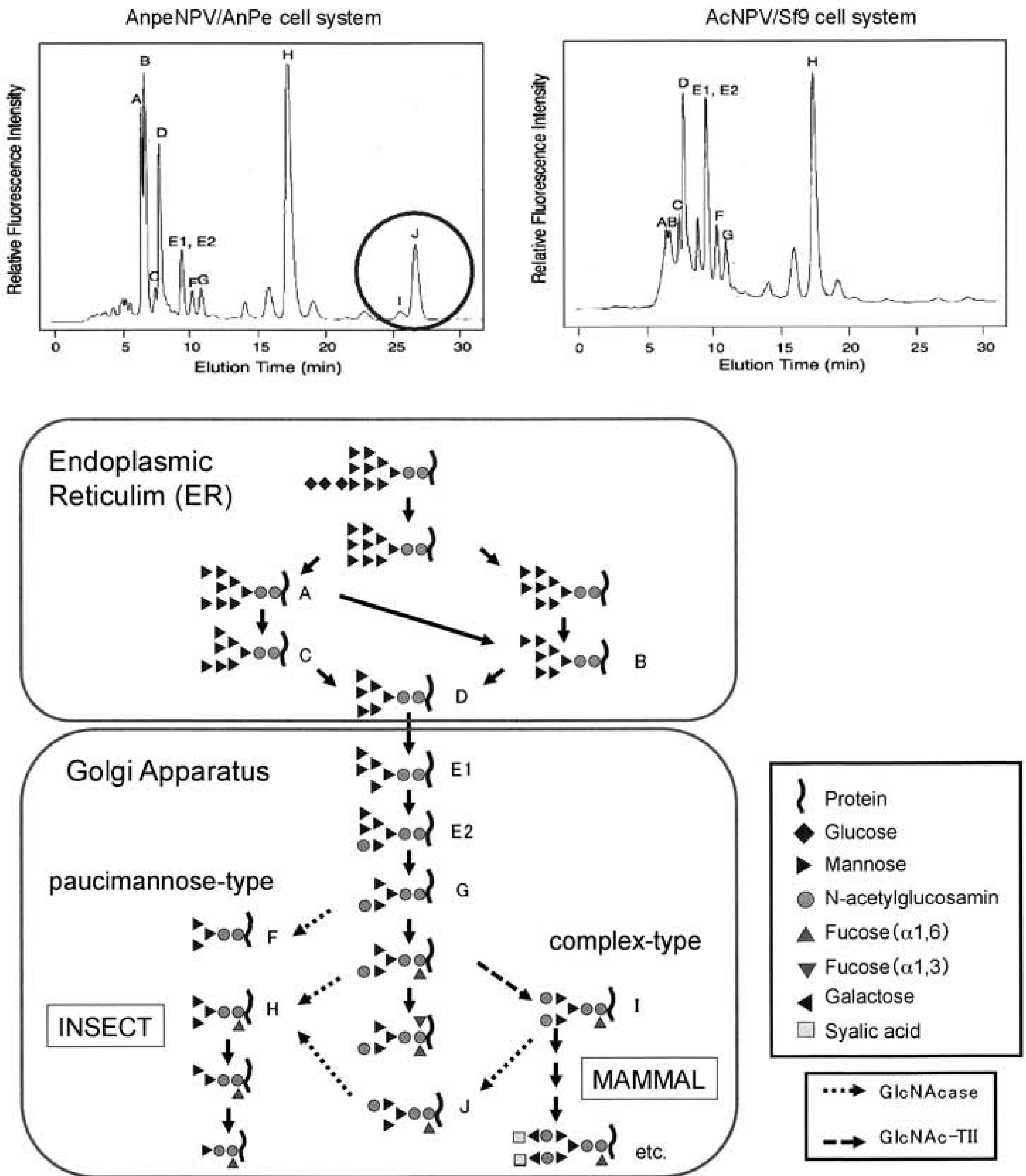


Fig. 4. Evaluation of *N*-glycosylation property of AnpeNPV/AnPe cell system by 2D sugar mapping of *N*-glycans derived from recombinant PTTH in comparison with that of AcNPV/Sf9 cell system (upper panel) and a predicted *N*-glycosylation pathway in AnPe cells (lower pane).

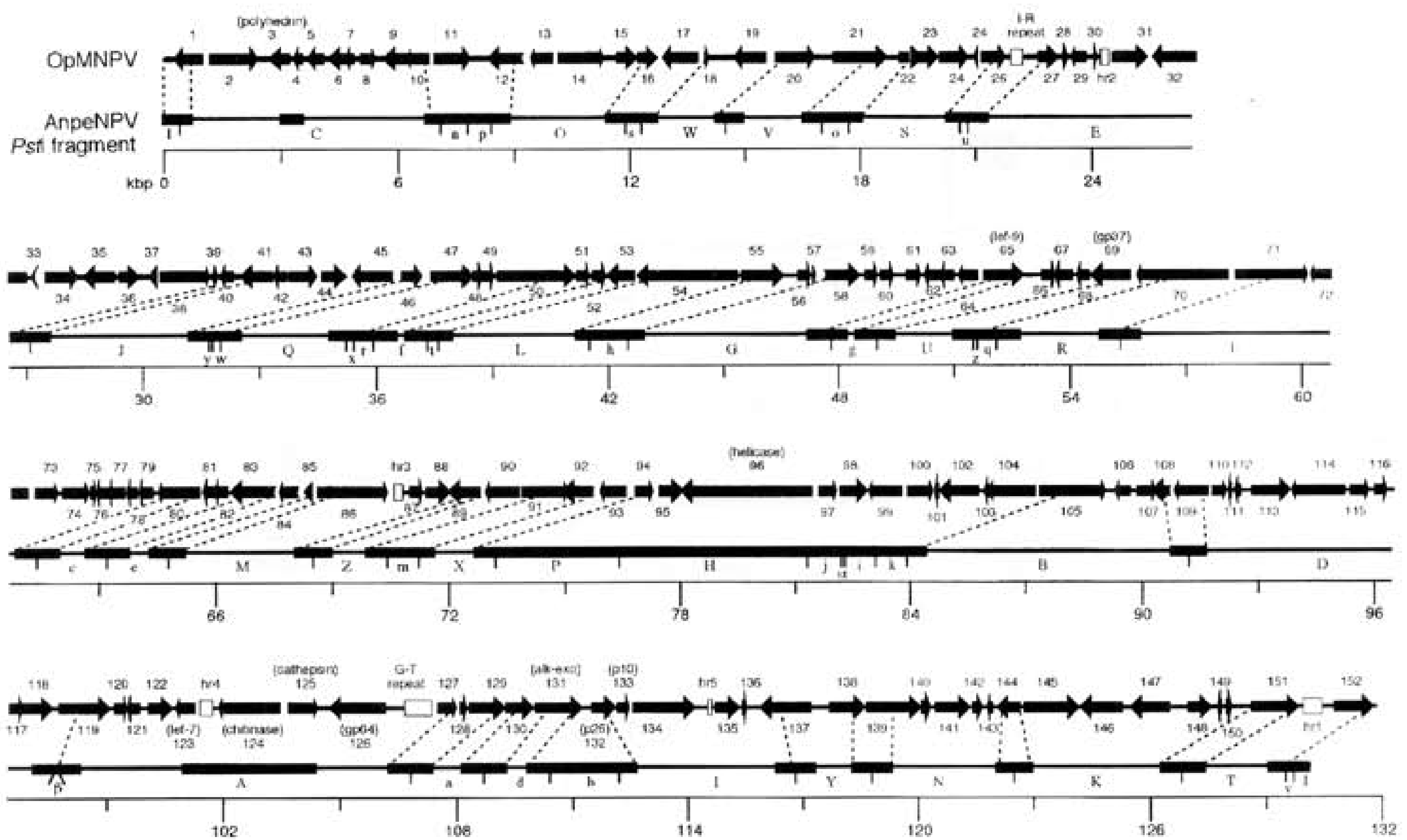


Fig. 5. The physical map of AnpeNPV clone A genome (lower line) constructed by aligning 54 *Pst*I fragments with closely related OpMNPV genome (upper line).

Table 1. Comparison of several statistics of the genome sequence between 3 isolates of AnpeNPV

	AnpeNPV		
	clone A	EF207986	NC_008035
Size in bp	126,592	126,246	126,629
GC Content (%)	53.4	53.5	53.5
No. of QRFs	150	145	147
Clockwise Orientation (ORF%)	48.7	46.9	48.3
No. of Hrs	6	6	3
No. of *bro* Genes	2	2	2

population (Table. 1).

Based on the absence of *Avr*II restriction site in the genome, two *Avr*II sites have been introduced at 5' and 3' UTRs of the polyhedrin gene for linearizing the AnpeNPV DNA. In addition, attR1 and attR2 sites for site-specific cloning of the gene of interest from Gateway® (Invitrogen) entry clone have been inserted just upstream and downstream of the two *Avr*II sites, respectively. The resulting viral DNA has allowed us to obtain recombinant virus by direct

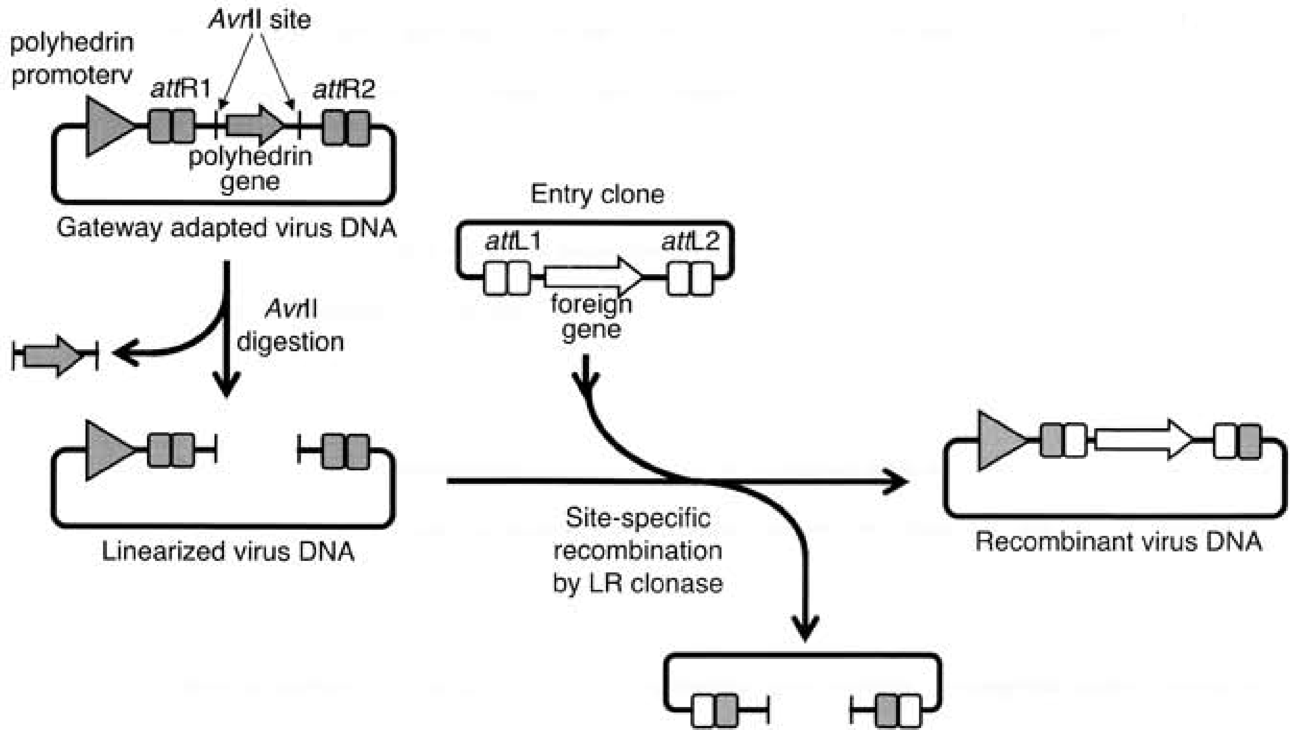


Fig. 6. Schematic representation of procedure for recombinant virus DNA construction in the Gateway-adapted AnpeNPV BEV system.

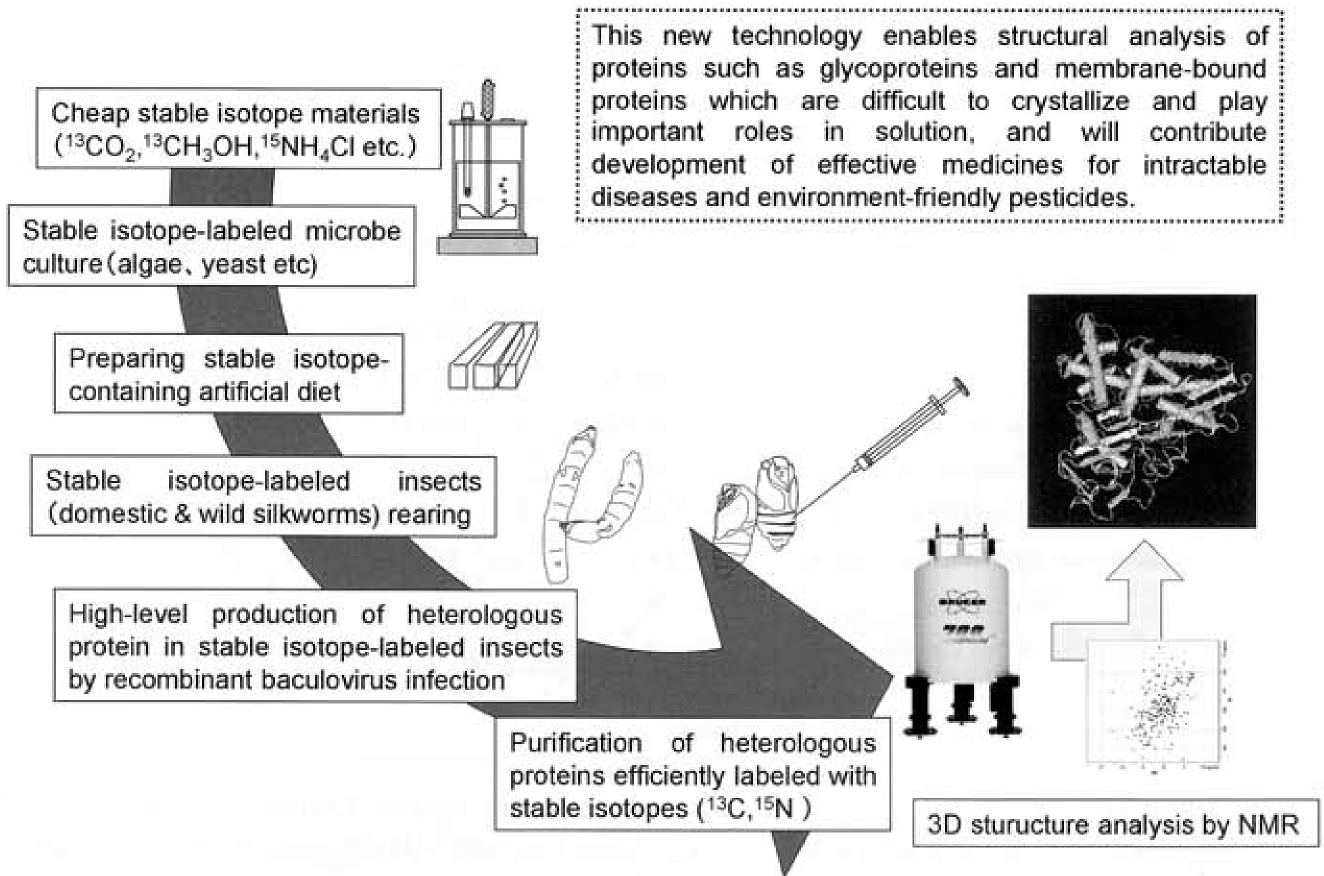


Fig. 7. Schematic representation of procedure for 3D protein structure analysis by *in vivo* stable isotope-labeling technology.

gene transfer in vitro without any purification steps (Fig. 6). In fact, desired recombinant viruses have been obtained with extremely high efficiencies (almost 100%) in many cases, demonstrating the virus vector applicable for high-throughput production of proteins.

Recently, we have developed a method to produce stable isotope (^{15}N, ^{13}C, etc.)-labeled recombinant proteins in larvae and pupae of both domestic and wild silkmoths (Kobayashi, 2007) (Fig. 7). This new technology will facilitate NMR-based structural analysis of proteins which do not easily crystallize and/or whose structures in water must be analyzed. The labeled diapausing pupae of wild silkmoths are storable for long periods and, thus, most suitable for hosts to produce labeled proteins cost effectively.

As demonstrated above, diapausing pupae of wild silkmoths used in the AnpeNPV BEV system are ideal bioreactors having long shelf lives more than one year and performing extremely high-level production of recombinant proteins without any nutritional inputs (Kobayashi, 2002). Further innovations will make this BEV system more useful tool in post-genome researches such as proteomics and metabolomics.

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