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Yamaji, Hideki

(Citation)

Cell Engineering, 7:53-76

(Issue Date)

2011

(Resource Type)

journal article

(Version)

Accepted Manuscript

(URL)

<https://hdl.handle.net/20.500.14094/90002563>



Production of Antibody in Insect Cells

Hideki Yamaji

Abstract Insect cells have proven to be an excellent platform for the production of recombinant antibodies. The baculovirus–insect cell system directs transient expression of recombinant antibodies in batch culture upon infection of insect cells with a recombinant baculovirus, while stably transformed insect cells allow constitutive or inducible production. Both systems provide rapid, simple ways of producing considerable amounts of recombinant antibody molecules with biological activities, in particular antibody fragments, such as scFv and Fab fragments, and their derivatives. Therefore, insect cell expression systems would be highly valuable for high-throughput antibody production. In addition, the display of heterologous proteins, including antibody fragments, on the baculovirus surface might be a useful tool for the generation and production of monoclonal antibodies with high affinity and specificity.

H. Yamaji

Department of Chemical Science and Engineering, Graduate School of Engineering,

Kobe University, 1–1 Rokkodai, Nada, Kobe 657–8501, Japan

e-mail: yamaji@kobe-u.ac.jp

Keywords: AcNPV, bacmid, baculovirus, baculovirus display, baculovirus–insect cell system, Fab, gp64, *N*-glycosylation, High Five, IgG, insect cell, metallothionein, multiplicity of infection, promoter, nucleopolyhedrovirus, paucimannose, polyhedrin, S2, scFv, secretory production, Sf9, signal peptide, stable transformation

1 Introduction

In recent years, insect cells have been used extensively as a platform for the production of a wide variety of biologically active recombinant proteins. Insect cells are easy to culture, as compared with mammalian cells. They can be maintained at 25–28 °C without CO₂ supplementation in the atmosphere. Insect cells have traditionally been cultured in basal media supplemented with around 10% vertebrate serum, most usually fetal bovine serum (FBS), instead of insect hemolymph. However, insect cells are easily adaptable to serum-free medium and can be grown to high densities in suspension culture. The doubling times of insect cells are typically 18–24 h, but the growth rate is slower at lower temperatures. Insect cells are capable of producing heterologous proteins through post-translational processing and modifications of higher eukaryotes. The baculovirus–insect cell system is the most commonly used insect cell-based expression system. Upon infection with a recombinant baculovirus carrying the foreign gene of interest, insect cells in culture often express large quantities of the foreign protein, which retains its functional activity during the very late stage of infection (Luckow, 1995). Recombinant baculoviruses have also been used for the production of various recombinant proteins in insect larvae or pupae (Kato et al., 2010).

In the baculovirus–insect cell system, recombinant protein is transiently expressed and commonly produced in batch culture, because continuous production is virtually impossible due to the lytic nature of the viral infection process. By contrast, recombinant protein can be synthesized continuously by integrating a constitutively expressed gene into the genome of a host cell line upon transfection with plasmid vectors. Stably transformed insect cell lines have been employed as an attractive

system for the continuous production of recombinant proteins (Douris et al., 2006; McCarroll and King, 1997; Pfeifer, 1998). The stably transformed insect cell system is especially useful for the production of secreted complex proteins, since the protein processing machinery and secretory pathway of the host insect cell are not damaged by baculovirus infection.

A display of heterologous peptides or proteins on the surface of virus particles is invaluable for the selection of genes that encode desired properties from combinatorial libraries based on protein interactions. In viral surface display systems, viruses are engineered to display foreign peptides or proteins on their surface by fusing the DNA encoding the peptides or proteins with a gene encoding a viral coat protein. The resulting recombinant viruses become vehicles that carry the DNA encoding expressed peptides or proteins, and replicate to high titers after infection of host cells. Using selection methods based on interactions between expressed peptides/proteins and target molecules, virus particles displaying peptides or proteins that bind specifically to the target are isolated from a large library of different expression clones, and specific virus clones can then be amplified in host cells. A phage display system, using filamentous bacteriophages that infect *Escherichia coli*, has proven to be a powerful tool for the selection of polypeptides with novel functions (Smith, 1985; Smith and Petrenko, 1997). Phage display, however, has limitations to the successful display of eukaryotic proteins that require complex folding and extensive post-translational processing and modifications. A variety of strategies have recently been developed for the display of heterologous proteins on the surface of baculovirus particles (Boublik et al., 1995; Grabherr et al., 2001; Mäkelä and Oker-Blom, 2006). Insect cells have been used as host cells for the generation and amplification of recombinant baculoviruses displaying

foreign proteins on their surface. Baculovirus display would be valuable in functional genomics and proteomics, and in drug discovery, because it allows the presentation of complex proteins through post-translational processing and modifications of higher eukaryotes on the viral surface.

The technologies mentioned above, using insect cells, are available for the generation and production of antibodies (Fig. 1). Like phage display, baculovirus display could be used to generate monoclonal antibodies that recognize a specific antigen by screening a library of baculoviruses expressing antibody molecules for the baculoviruses that bind to the target antigen. Meanwhile, baculovirus surface display has been employed for the generation of monoclonal antibodies by immunizing mice with baculoviruses displaying protein immunogens (Lindley et al., 2000; Mäkelä and Oker-Blom, 2006). Furthermore, the baculovirus–insect cell system and the stably transformed insect cell system have been widely used for the efficient expression of antibody genes. This chapter gives an overview of these insect cell-based technologies for the generation and production of recombinant antibody molecules (Fig. 1).

2 Antibody Production in the Baculovirus–Insect Cell System

The baculovirus–insect cell system is the most widely established and commonly used insect cell-based expression system, as it can produce large amounts of the foreign protein of interest. Baculoviruses are a family of rod-shaped viruses with a circular double-stranded DNA genome, which exclusively infects invertebrates (mainly insects). The baculoviruses commonly used in the baculovirus–insect cell system are in the genus *Nucleopolyhedrovirus*, such as *Autographa californica* nucleopolyhedrovirus

(AcNPV) and *Bombyx mori* nucleopolyhedrovirus (BmNPV), which are pathogenic for lepidopteran insects such as butterflies and moths. Three insect cell lines derived from lepidopteran insects are most commonly used with AcNPV in the baculovirus–insect cell system. The Sf21 cell line and its clonal isolate Sf9 are derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda*. The BTI-TN-5B1-4 (High Five) cell line originated from the ovarian cells of the cabbage looper, *Trichoplusia ni*.

Upon infection of a susceptible insect with a nucleopolyhedrovirus, progeny virus particles are released by budding off from the cell membrane of infected cells during the early phase of infection, and then these particles spread the infection throughout the insect. During the very late stage of infection, infected cells synthesize immense quantities of a viral protein called polyhedrin (Fig. 2). Polyhedrin molecules assemble into large particles called occlusion bodies or polyhedra, in which multiple progeny virions are embedded. These particles are released into the environment for horizontal transmission of the virus after cell lysis and the death of a host insect. Polyhedrin is important in the life cycle of a wild-type baculovirus because it protects the embedded virus particles from inactivation by environmental factors until ingestion by a susceptible insect. The promoter of the nucleopolyhedrovirus polyhedrin gene is remarkably strong. However, the polyhedrin gene is not essential for infection or for replication of the virus. In the baculovirus–insect cell system, therefore, a recombinant nucleopolyhedrovirus is constructed, in which the polyhedrin gene is usually replaced with the foreign gene of interest. Subsequent infection of cultured lepidopteran insect cells with the recombinant baculovirus often leads to the expression of extremely large quantities of the foreign protein, instead of polyhedrin, under the control of the very

strong polyhedrin promoter (Fig. 2). The strong and “very late” promoter of the nucleopolyhedrovirus p10 gene has also been used in the baculovirus–insect cell system.

Baculovirus-infected insect cells perform most of the post-translational processing and modifications of higher eukaryotes, including phosphorylation, glycosylation, correct signal peptide cleavage, proteolytic processing, and fatty acid acylation (Luckow, 1995). The highly restricted host range of the baculovirus makes this expression system safe. A wide variety of recombinant proteins, including both secreted and membrane-bound proteins, have been successfully produced in the baculovirus–insect cell system (Luckow, 1995; Massotte, 2003). With respect to glycosylation, insect cells can transfer oligosaccharide side chains to the same sites in recombinant proteins as those in native mammalian proteins. However, insect cells mostly produce simpler *N*-glycans with terminal mannose residues, paucimannose *N*-glycans, while mammalian cells produce more complex *N*-glycans containing terminal sialic acids. This difference in the *N*-glycosylation between insect and mammalian cells may limit the application of recombinant glycoproteins produced in insect cells to therapeutics for human use, although recent genetic and metabolic engineering of the insect *N*-glycosylation pathway has been shown to successfully produce complex, terminally sialylated *N*-glycans (Harrison and Jarvis, 2006; Kost et al., 2005; Tomiya et al., 2004).

Recombinant baculoviruses have traditionally been constructed based on *in vivo* inefficient homologous recombination between a transfer vector carrying the foreign gene of interest and the nucleopolyhedrovirus DNA genome, both of which are co-transfected into cultured insect cells (Fig. 2, middle panel). The resulting recombinant virus progeny are isolated from wild-type parental baculoviruses by time-consuming and subjective plaque assay with microscopic observation. This traditional procedure

for the generation of a recombinant baculovirus has been improved to simplify the overall process, and rapid and efficient methods to generate recombinant baculovirus have been developed (Jarvis, 2009; Kost et al., 2005; Luckow, 1995). These include an approach using site-specific transposon-mediated insertion of the gene of interest into a baculovirus genome (bacmid) in *E. coli* (Luckow et al., 1993). This system is commercially available as the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA). A faster and easier method for direct transfer of the gene of interest into the baculovirus genome *in vitro*, based on bacteriophage lambda site-specific recombination, has also been developed and is commercially available as the BaculoDirect baculovirus expression system (Invitrogen).

A large number of intact immunoglobulins in a biologically active form have been expressed at high yields using the baculovirus–insect cell system (Edelman et al., 1997; Hasemann and Capra, 1990; Liang et al., 1997, 2001; Nesbit et al., 1992; Poul et al., 1995; Song et al., 2010; Tan and Lam, 1999; zu Putlitz et al., 1990) (Table 1). Functional Fab fragments (Abrams et al., 1994; Furuta et al., 2010) and scFv fragments (Brocks et al., 1997; Kretzschmar et al., 1996; Laroche et al., 1991; Lemeulle et al., 1998; Yoshida et al., 1999) have also been expressed successfully in the baculovirus–insect cell system. Fab fragments consist of two chains comprised of the variable and the first constant domains of an immunoglobulin, VH + CH1 (Fd fragment) and VL + CL (light chain), which need to assemble with a disulphide bond (Fig. 3). On the other hand, scFv fragments are contiguous polypeptides that consist of the variable heavy and light chain domains (VH, VL) of an immunoglobulin, linked through a flexible peptide linker such as (Gly₄Ser)₃. Whereas scFv fragments can be efficiently produced in *E. coli*, expressed proteins often form insoluble aggregates called inclusion bodies in the

cytoplasm, which requires proper refolding of the inactive proteins. In contrast, insect cells effectively secrete scFv fragments with antigen-binding activity into the extracellular fluid. Comparable or higher yields of scFv fragments were achieved in baculovirus-infected insect cells as compared with *E. coli* cells (Brocks et al., 1997; Lemeulle et al., 1998). Furthermore, the baculovirus–insect cell system has been used to express scFv fusion proteins (Bei et al., 1995; Brocks et al., 1997; Peipp et al., 2004) and bispecific scFv antibodies (Bruenke et al., 2004; Yoshida et al., 2003a).

To express an intact IgG and an Fab fragment, the genes that encode both the heavy chain and the light chain must be expressed in the same cell. Although expression of both the heavy- and light-chain genes can be achieved by double infection of insect cells with two recombinant baculoviruses, one carrying the heavy chain gene and the other the light chain gene (Abrams et al., 1994; Hasemann and Capra, 1990; Nesbit et al., 1992), this strategy requires the generation of two recombinant baculoviruses and careful adjustment of the infection conditions. Therefore, recombinant baculoviruses that contain both the heavy- and light-chain cDNA have been generated and used for the expression of intact IgG molecules (Liang et al., 1997, 2001; Song et al., 2010; Tan and Lam, 1999; zu Putlitz et al., 1990) and an Fab fragment (Furuta et al., 2010).

Prior to the generation of a recombinant baculovirus, factors, such as the promoter and the signal peptide, should be considered, because they affect the expression level of the recombinant protein. Various dual-expression vectors (Luckow, 1995), which are designed to accept and express two foreign genes, are available for the generation of a single recombinant baculovirus that contains both the heavy- and light-chain genes. When a dual-expression vector that has two different promoters is employed, the expression level may depend on the combination of promoter and gene. For the

production of an Fab fragment, Furuta et al. (2010) generated recombinant baculoviruses using the Bac-to-Bac baculovirus expression system (Invitrogen) with the donor plasmid pFastBac Dual, which contains two multiple cloning sites to allow simultaneous expression of two heterologous genes in a single recombinant baculovirus. In the pFastBac Dual, one gene is expressed under the control of the AcNPV polyhedrin promoter, while the other is under the control of the AcNPV p10 promoter. Both the polyhedrin and p10 promoters are strong and “very late” promoters, but the promoter (polyhedrin or p10) and gene (heavy chain or light chain) combination may affect the expression level of the Fab fragment. High Five cells infected with a recombinant baculovirus, in which the light- and heavy-chain genes were located downstream of the p10 and polyhedrin promoters, respectively, produced a higher Fab fragment yield than those with a baculovirus in which the heavy- and light-chain genes were downstream of the p10 and polyhedrin promoters, respectively (Furuta et al., 2010).

Signal peptides that target secreted proteins into the endoplasmic reticulum and through the secretory pathway may influence the production of antibody molecules by baculovirus-infected insect cells. DNA sequences encoding authentic signal peptides of the heavy chain and the light chain have been used (Hasemann and Capra, 1990; Liang et al., 1997, 2001; Nesbit et al., 1992; Song et al., 2010), because the signal peptides are properly processed in the baculovirus-infected insect cells (Hasemann and Capra, 1990). Signal peptides of insect proteins like honeybee melittin (Abrams et al., 1994; Tan and Lam, 1999) and *B. mori* larval serum protein (Tan and Lam, 1999) have also been employed for the production of an IgG and an Fab fragment. Furuta et al. (2010) compared the effects of three different signal peptides of honeybee melittin, *Drosophila* BiP, and AcNPV gp64 on the secretory production of a mouse Fab fragment

by baculovirus-infected High Five cells. The use of the DNA sequence encoding baculovirus gp64 signal peptide upstream of the heavy- and light-chain genes resulted in a higher yield of the secreted Fab fragment than the sequences of insect-derived BiP and melittin signal peptides, but there were no notable differences in the secretion efficiency of the different signal peptides. A signal peptide from honeybee melittin has been successfully used to express an scFv fusion protein (Peipp et al., 2004) and bispecific scFv antibodies (Bruenke et al., 2004; Yoshida et al., 2003a) as secreted proteins, while scFv fragments linked to this signal peptide were not secreted into a culture medium (Reavy et al., 2000; Yoshida et al., 1999). A signal peptide from AcNPV gp64 has been employed for the secretory expression of an scFv fragment (Kretzschmar et al., 1996). In addition, a monovalent scFv and a bivalent scFv fusion protein with a human IgG1 Fc region were expressed and secreted after correctly processing a signal peptide from human IL-6 receptor (Brocks et al., 1997).

Once a recombinant baculovirus has been generated, the recombinant protein can be obtained upon infection of cultured insect cells. Batch culture is generally employed for recombinant protein production by the baculovirus–insect cell system, because of the lytic nature of the viral infection process. Production of antibody molecules, as well as other recombinant proteins, is affected by a number of factors, which include host insect cells, culture medium, culture method, dissolved oxygen concentration, the multiplicity of infection (MOI), and cell density at the time of infection (Taticek et al., 1995). High Five cells have been shown to be better for the expression of secreted recombinant proteins than Sf9 or Sf21 cells (Davis et al., 1992; Taticek et al., 2001; Wickham and Nemerow, 1993), but exceptions have also been reported (Chai et al., 1996; Rhiel et al., 1997; Sugiura and Amann, 1996). Baculovirus-infected High Five

cells in serum-free medium Express Five (Invitrogen) produced a higher Fab fragment yield than Sf9 cells in TNM-FH supplemented with 10% FBS, in both static and shake-flask cultures (Furuta et al., 2010). When infection is performed at high cell densities, recombinant protein production in batch culture is often limited by nutrient depletion in the culture medium (Radford et al., 1997; Taticek and Shuler, 1997; Yamaji et al., 1999). Hence, it is important to choose an appropriate combination of MOI and cell density at the time of infection, so that the recombinant protein production is completed before nutrient depletion in the culture medium occurs (Power et al., 1994; Wong et al., 1996; Yamaji et al., 1999; Yang et al., 1996).

The expression of a functional IgG may be accompanied by the accumulation of insoluble immunoglobulin aggregates in baculovirus-infected insect cells (Hasemann and Capra, 1990), indicating a limitation in the processing and secretory pathway of the insect cells. In this case, co-expression of an enzyme protein disulfide isomerase (PDI), an endoplasmic reticulum molecular chaperone BiP, or a cytosolic chaperon hsp70 may improve the solubility and secretion of recombinant IgG in the baculovirus–insect cell system, as previously reported (Alior and Betenbaugh, 1998; Hsu and Betenbaugh, 1997; Hsu et al., 1996).

Because a recombinant baculovirus can also infect insect larvae or pupae, they have been used for recombinant protein production as “protein factories” (Kato et al., 2010; Maeda et al., 1985; Medin et al., 1990). Upon infection of silkworm larvae using a needle moistened with a recombinant BmNPV, a functional IgG has been expressed at a concentration of 800 µg/ml in the hemolymph (Reis et al., 1992) (Table 2). A functionally active Fab fragment has been produced, with a yield of 1.1 mg/g-larvae, upon infection of *T. ni* larvae after the ingestion of a diet containing a recombinant

AcNPV (O'Connell et al., 2007). More recently, an scFv fragment and an IgG have been produced rapidly in silkworm larvae and pupae injected with recombinant BmNPV bacmid (Dojma et al., 2010; Ishikiriya et al., 2009; Park et al., 2009; Sakamoto et al., 2010). A significantly high level expression of recombinant antibodies can be achieved in insect larva and pupae. Whereas an automated insect rearing system and a method for the oral infection of larvae with recombinant baculovirus have been developed (O'Connell et al., 2007), efficient methods for the injection of bacmids into insects and for the recovery and purification of products from insects should be developed for the large-scale production of recombinant antibodies.

3 Antibody Production in Recombinant Insect Cells

Continuous protein production provides good lot-to-lot reproducibility and facilitates large-scale production in bioreactors. In the baculovirus–insect cell system, however, continuous protein production is virtually impossible because of the lytic nature of the viral infection process. The lysis of host insect cells following baculovirus infection, and the resultant release of intracellular proteins, may also result in protein degradation by proteases and the need for complicated downstream processing and purification of products. Proteolysis control in the baculovirus–insect cell system has therefore been investigated, including the addition of protease inhibitors and the use of baculoviruses that lack protease genes (Gotoh et al., 2001a, b; Ikonomou et al., 2003).

Stably transformed insect cell lines have emerged as attractive alternative platforms for the continuous production of complex recombinant proteins (Douris et al., 2006; McCarroll and King, 1997; Pfeifer, 1998). In the stably transformed insect cell system,

host insect cells are transfected with a plasmid vector into which the foreign gene of interest is cloned under the control of an appropriate promoter (Fig. 4). If the introduced vector integrates into the chromosomal DNA of the host cell, the foreign protein can be synthesized either constitutively or upon induction. In order to identify a small fraction of the stably transformed cells, antibiotic resistance genes are used as selectable markers and are co-transfected together with the heterologous gene of interest. This system is especially useful for the production of secreted complex proteins, as the protein synthesis and processing machinery of the host insect cell is not compromised by baculoviral infection.

In the stably transformed insect cell system, cell lines derived from dipteran insects, such as fruit flies and mosquitoes, have often been employed as host cells. In particular, the *Drosophila melanogaster* Schneider 2 (S2) cell line has been most commonly used for inducible expression with the *Drosophila* metallothionein promoter. An antibiotic resistance gene, such as a hygromycin, blasticidin, or neomycin resistance gene, has been used as a selectable marker and co-transfected with the heterologous gene of interest downstream of the metallothionein promoter. Hundreds of copies of the expression plasmid stably integrate into the genome of *Drosophila* S2 cells in a single transfection-selection event (Johansen et al., 1989). The metallothionein promoter is tightly regulated at high copy numbers, and is capable of directing high levels of expression when induced by copper sulfate (Kirkpatrick and Shatzman, 1999). After co-transfection of the heavy- and light-chain cDNA with an antibiotic resistance gene, human and humanized monoclonal antibodies with antigen-binding activity have been efficiently expressed and secreted under the control of the *Drosophila* metallothionein promoter in stably transformed *Drosophila* S2 cells (Johansson et al.,

2007; Kirkpatrick et al., 1995) (Table 3). Secretory production of functional scFv fragments has also been achieved in recombinant S2 cells (Mahiouz et al., 1998; Reavy et al., 2000). In addition, constitutive secretory expression of a biologically active scFv fragment using the *Drosophila* actin 5C promoter in S2 cells has been demonstrated (Gupta et al., 2001).

Stable transformation of lepidopteran insect cells, such as Sf9 and High Five cells, has been investigated as well. In this case, the choice of a promoter to drive the heterologous gene expression is important, as the use of weak promoters results in low recombinant protein yields. Reportedly, the protein yields in stably transformed lepidopteran cells have often been considerably lower than those obtained in the baculovirus–insect cell system (Ivanova et al., 2007; Jarvis et al., 1990). Guttieri et al. (2000) separately cloned the heavy- and light-chain genes of a monoclonal antibody downstream of the AcNPV hr5 enhancer and the AcNPV immediate early promoter IE1 in two plasmid vectors. High Five cells were then co-transfected with the constructs and a selection plasmid carrying a neomycin resistance gene. After selection, stably transformed cells continuously secreted biologically active IgG, although the yield of IgG in the culture supernatant was considerably lower (0.06 µg/ml) than that obtained in the baculovirus–insect cell system (9 µg/ml) (Table 3). Li et al. (2001) cloned immunoglobulin heavy- and light-chain genes downstream of the *Orgyia pseudotsugata* NPV immediate early 2 promoter OpIE2 in two plasmid vectors containing a Zeocin resistance gene. Stably transformed Sf9 cells secreted functional antibodies at yields of approximately 1 µg/ml.

Recently, the expression vector pIE1/153A has been developed for the continuous high-level expression of secreted proteins by transformed lepidopteran insect cells

(Farrell et al., 1998, 1999; Keith et al., 1999). pIE1/153A utilizes the *B. mori* cytoplasmic actin promoter, from which foreign gene expression is stimulated with the BmNPV IE-1 transactivator and the BmNPV HR3 enhancer. Use of the IE-1 transactivator and the HR3 enhancer has resulted in a more than 1,000-fold increase in the stimulation of foreign gene expression through the actin promoter (Lu et al., 1997). Lepidopteran insect cells are first co-transfected with pIE1/153A containing the heterologous gene and a plasmid vector carrying a selectable marker, after which antibiotic selection and isolation of highly productive clones is carried out. Stable cell lines expressing secreted proteins, including tissue plasminogen activator and secreted alkaline phosphatase, have shown higher expression levels than the baculovirus–insect cell system (Farrell et al., 1999; Jardin et al., 2007; Kato et al., 2004).

When the expression vector mentioned above, utilizing the BmNPV IE-1 transactivator, the BmNPV HR3 enhancer, and the *B. mori* actin promoter, is employed for the expression of an intact IgG or an Fab fragment (Fig. 3), host insect cells must be co-transfected with a total of three plasmid vectors: two sets of expression vectors that separately contain the genes encoding the heavy chain and light chain of the antibody molecule, and a plasmid carrying a selectable marker. Co-transfection with three plasmid vectors inevitably results in low efficiency in obtaining stably transformed cells producing the antibody molecule. On the basis of the expression vector utilizing the IE-1 transactivator, the HR3 enhancer, and the actin promoter, Yamaji et al. (2008) constructed two plasmid vectors, pIHAbLa and pIHAneo, which contain either a blasticidin or a neomycin resistance gene, for use as a selectable marker (Fig. 5). After co-transfection with these plasmid vectors, into which the heavy- and light-chain genes of an Fab fragment were separately inserted, High Five cells secreting a high

concentration of the Fab fragment were efficiently generated by incubation in the presence of blasticidin and G418. High yields of more than 300 µg/ml of the Fab fragment were produced in a shake-flask culture of the recombinant insect cells.

Transgenic silkworms that synthesize a functional IgG into the sericin layer of silk fibers in their cocoons have recently been generated (Iizuka et al., 2009) (Table 2). The IgG produced was extractable from the cocoons with a buffer containing 3 M urea.

4 Baculovirus Display for Antibody Generation

Phage display has been successfully used to select antibodies that recognize specific antigens from diverse libraries (Bradbury and Marks, 2004; McCafferty et al., 1990). In antibody phage display, antibody fragments, such as scFv fragments and Fab fragments, are expressed on the surface of filamentous bacteriophages by fusion of the genes encoding the antibody fragments to one of the phage coat proteins. Specific antibody-expressing phages can then be amplified in *E. coli* after multiple rounds of affinity selection for the antigen. When displayed on the phage surface, Fab fragments have a tendency to be more functional than the corresponding scFv fragments; some scFv fragments show a lower affinity than the corresponding Fab fragments (Bird and Walker, 1991; Bradbury and Marks, 2004). However, Fab fragments are often produced at significantly lower yields in *E. coli*, because they are twice the size of scFv and require the assembly of two protein chains.

Recently, baculoviruses have been successfully used for the display of heterologous proteins on the surface of viral particles by fusing the protein to the major baculoviral envelope glycoprotein gp64 (Boublik et al., 1995; Grabherr et al., 2001; Mäkelä and

Oker-Blom, 2006). gp64, also known as gp67, is essential for viral entry into insect cells via baculovirus-cell fusion. During the infection cycle, gp64 is abundantly expressed and transported to the surface of infected cells for incorporation into budding virions. In a typical baculovirus display, a recombinant baculovirus is constructed so that the gene of a target protein is inserted between the signal sequence and the mature protein domain of a second copy of the gp64 gene to anchor the fusion protein in the envelope of the baculovirus via the transmembrane region of gp64. Upon infection of insect cells with such a recombinant baculovirus, gp64 fused to the target protein, as well as wild-type gp64, are expressed and transported to the cell membrane, where they are picked up by progeny viruses during the budding process, thereby displaying the gp64-fusion protein on the surface of viral particles. Alternative baculovirus surface display strategies have also been developed (Grabherr et al., 2001; Mäkelä and Oker-Blom, 2006). Baculovirus display allows the presentation of large complex proteins with the eukaryotic posttranslational modification of insect cells.

Murine scFv fragments specific to the hapten 2-phenyloxazolone and human scFv against carcinoembryonic antigen were successfully displayed in a functional form on the AcNPV surface by fusion to the N-terminus of gp64 (Mottershead et al., 2000; Ojala et al., 2001). These results suggest that baculovirus particles displaying scFv fragments could be useful in targeting to a specific antigen for gene or drug delivery, although there would be little advantage to the use of baculoviruses displaying scFv fragments for the selection of specific antibodies, because scFv phage display has proven effective.

Generation of a recombinant baculovirus displaying antibody Fab fragments on its surface has been investigated (Yamaji and Fukuda, 2007). A recombinant baculovirus

was designed so that either the heavy chain (Fd fragment) or the light chain of a mouse Fab fragment (Fig. 3) was expressed as a fusion protein to the N-terminus of AcNPV gp64, while at the same time the other chain of the Fab fragment was expressed as a secretion protein. For expression as a gp64-fusion protein, the cDNA fragment encoding the heavy- or light-chain gene of the Fab fragment was inserted between the signal sequence and the mature protein domain of a second copy of the AcNPV gp64 gene. On the other hand, the *Drosophila* BiP signal sequence was employed upstream of the light- or heavy-chain gene for secretory expression. The resulting DNA fragments were PCR-cloned into a donor plasmid, pFastBac Dual (Invitrogen), which contained two multiple cloning sites. A recombinant baculovirus was then generated using the Bac-to-Bac baculovirus expression system (Invitrogen). When the culture supernatant, after infection of Sf9 cells with the recombinant baculovirus, was analyzed by enzyme-linked immunosorbent assay (ELISA) using antigen-coated 96-well plates and either an anti-AcNPV gp64 antibody or an anti-mouse IgG, a relatively strong signal showing antigen-binding activity was obtained in each case. In western blot analysis of the culture supernatant under reducing conditions, a specific protein band was detected at an electrophoretic mobility coinciding with the molecular weight of the gp64-fusion protein. The Fab fragments were successfully detected on the surface of Sf9 cells infected with the recombinant baculovirus by flow cytometry using fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (Fig. 6). These results suggest that antibody Fab fragments can be displayed on the surface of baculovirus particles in a functional form, and that baculoviruses displaying target Fab fragments could be selected using a fluorescence-activated cell sorter (FACS) with a fluorescence-labeled antigen. The use of baculovirus display technologies for the generation and screening

of cDNA expression libraries has been successfully demonstrated (Crawford et al., 2004, 2006; Ernst et al., 1998), although the methods have not yet been applied to antibody selection. While further developments are needed, baculovirus display of antibody molecules, including Fab fragments, may provide an opportunity for the selection of functional antibodies with high affinity and specificity.

Baculoviruses displaying gp64-fusion proteins have been successfully used as effective immunogens for the generation of monoclonal antibodies (Lindley et al., 2000; Mäkelä and Oker-Blom, 2006). Lindley et al. (2000) constructed recombinant baculoviruses, which displayed N-terminal domains of human nuclear receptors LXR β and FXR on the surface, by insertion of the corresponding gene fragment between the signal sequence and the mature protein domain of the AcNPV gp64 gene. After recombinant baculoviruses were pelleted by ultra-centrifugation and filter sterilized, mice were immunized with the whole baculovirus displaying respective gp64/nuclear receptor fusion proteins to produce hybridoma cells. Monoclonal antibodies that recognized LXR β or FXR were successfully generated with a low background of monoclonal antibodies against viral proteins, except for gp64. This method allows for the rapid production of immunogens without rigorous purification once the DNA sequence is available. Since fusion proteins are displayed on the viral surface in a native form, due to the similarities in protein processing and post-translational modification between insect and mammalian systems, this method may enhance the generation of antibodies capable of recognizing antigens under non-denaturing conditions (Lindley et al., 2000). Similarly, baculoviruses displaying gp64-fusion proteins have been successfully used to generate monoclonal antibodies against human peroxisome proliferator-activated receptors (Tanaka et al., 2002). Baculovirus-

displaying proteins, including *Plasmodium berghei* circumsporozoite protein (Yoshida et al., 2003b), foot-and-mouth disease virus antigen (Tami et al, 2004), bovine herpesvirus-1 glycoprotein D (Peralta et al., 2007), avian influenza virus hemagglutinin (Yang et al., 2007), and the E2 envelope glycoprotein of classical swine fever virus (Xu and Liu, 2008), have also proven to effectively elicit protective immune responses in mice, indicating that baculovirus display could be useful for the development of potential vaccine candidates.

5 Conclusions and Future Developments

A variety of insect cell-based technologies are currently available for the generation and production of antibodies (Fig. 1). Baculoviruses displaying foreign proteins on their surface have proven useful as effective immunogens for the immunization of mice to obtain hybridoma cells that produce monoclonal antibodies. The display of antibody Fab fragments on the baculovirus surface might also offer a novel approach for the selection of monoclonal antibodies with high affinity and specificity from diverse libraries. Insect cell-based expression systems, both the well developed baculovirus–insect cell system and the relatively new stably transformed cell system, provide a rapid, simple way to produce considerable amounts of recombinant antibody molecules that retain their functional activities (Tables 1 and 3). Optimization of the generation of recombinant baculovirus and stably transformed insect cells, and further development of culture media and cell lines, will lead to higher yields of recombinant antibodies in these systems. High-throughput antibody production in insect cell systems would have particular value for the discovery and development of efficacious therapeutic antibody

leads, and the development of antibody microarrays in proteomics and clinical diagnostics. Advances have been made in the development of bioprocesses for the large-scale, high-density culture of insect cells (Chan et al., 1998; Chico and Jäger, 2000; Ikonomou et al., 2003; Jardin et al., 2007; Marteiijn et al., 2003; Taticek et al., 1995; Yamaji et al., 2006), which also result in increased yields of recombinant antibodies. With respect to biomedical applications of insect cell expression systems, the baculovirus–insect cell system is employed for the industrial-scale production of a human papillomavirus vaccine, Cervarix, which has been approved for use against cervical cancer (Schiller et al., 2008). In the case of recombinant antibodies, however, the paucimannose type *N*-glycosylation typically observed in insect cells may limit the use of whole antibodies produced in insect cells as therapeutics for human use, while the engineering of the insect *N*-glycosylation pathway has been shown to allow the production of mammalianized *N*-glycoproteins (Harrison and Jarvis, 2006; Kost et al., 2005; Tomiya et al., 2004). For the present, therefore, insect cells can serve as a platform for the efficient production of antibody fragments, such as scFv and Fab fragments, and related proteins, but not intact IgG, for use as therapeutic applications. Future developments in insect cell and baculovirus engineering will provide further opportunities for enhancement of the utility and applications of recombinant antibodies produced in insect cell expression systems.

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Figure captions

Fig. 1 Generation and production of antibodies using insect cell-based technologies

Fig. 2 Recombinant protein production in the baculovirus–insect cell system

Fig. 3 Schematic representation of an antibody molecule (human IgG1) and its fragments. Fab fragments can be made by papain digestion of an IgG molecule. Single-chain Fv (scFv) fragments are a recombinant molecule consisting of the VH and VL domains of an IgG molecule, linked through a flexible peptide linker

Fig. 4 Recombinant protein production by stably transformed insect cell lines

Fig. 5 Expression vectors for stable transformation of lepidopteran insect cells

Fig. 6 Flow cytometric analysis of Sf9 cells infected with a recombinant baculovirus displaying mouse Fab fragments on its surface. Recombinant baculovirus carrying the genes of the Fd fragment and the light chain-gp64 fusion was used. Infected cells 24 h post-infection and uninfected cells were treated with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG before flow cytometry

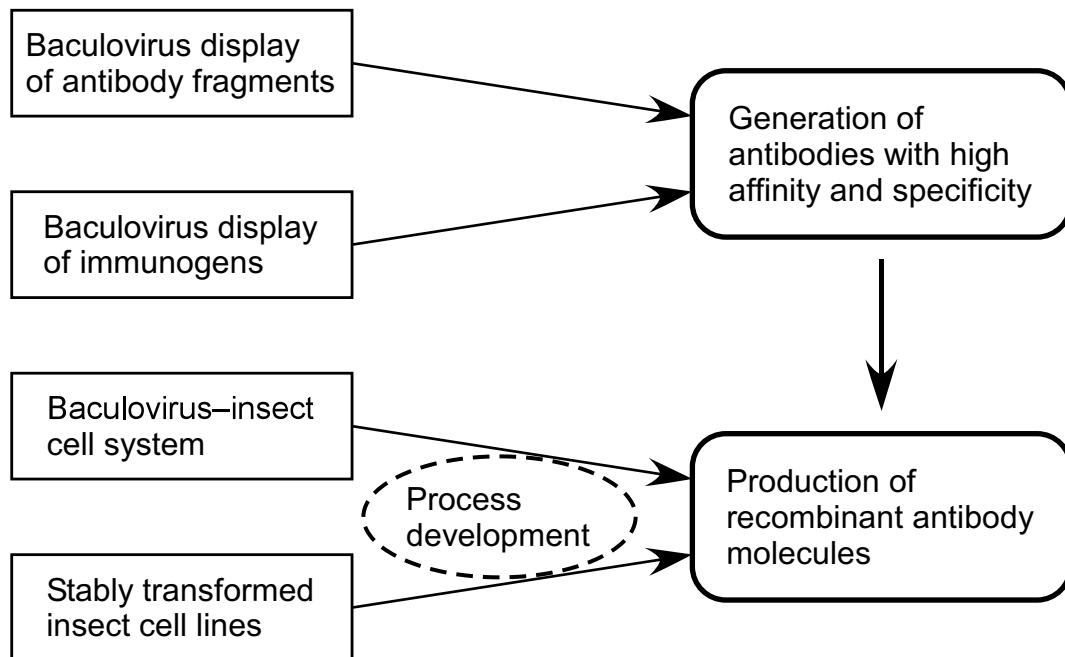


Fig. 1 Generation and production of antibodies using insect cell-based technologies

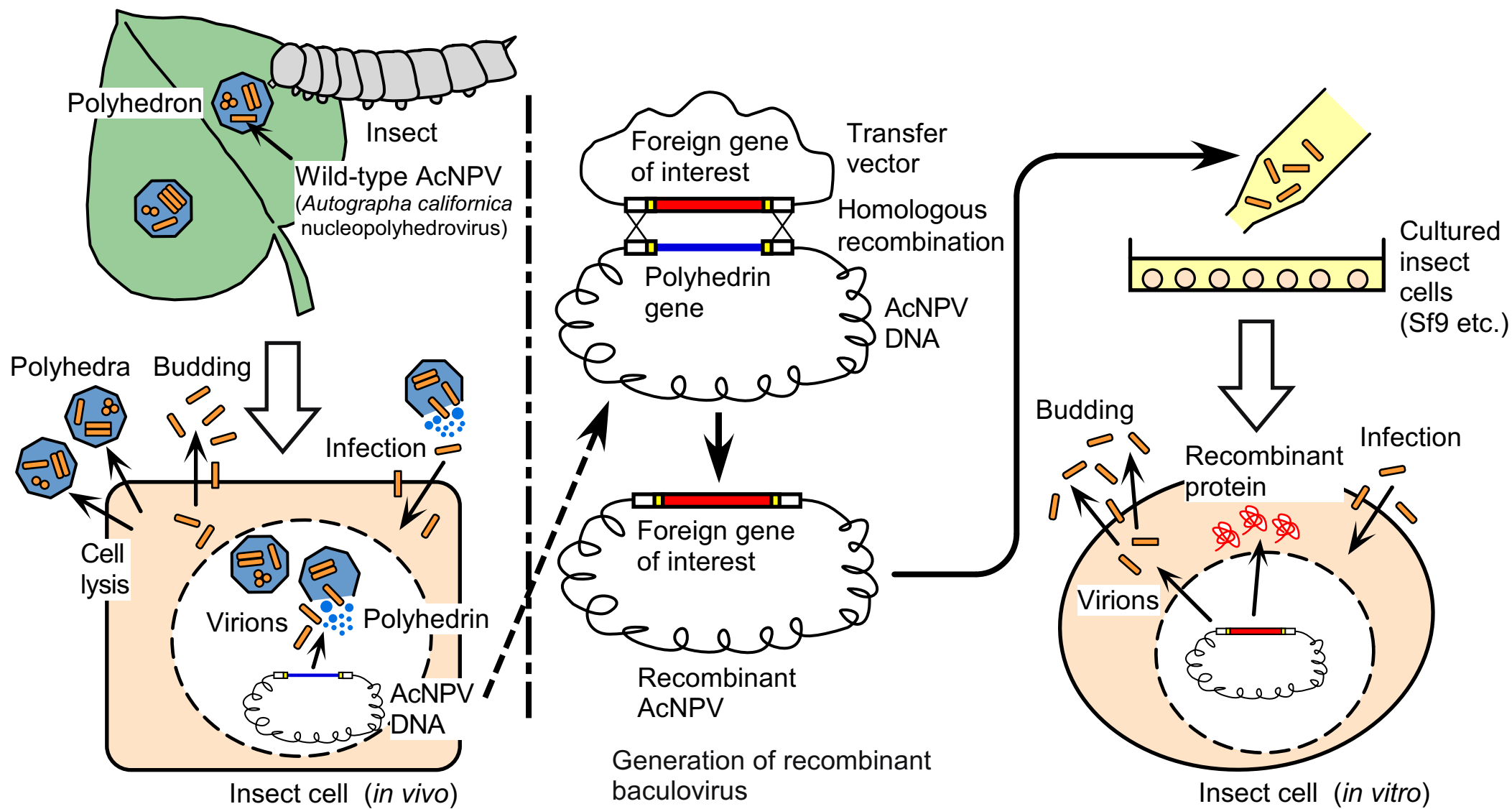


Fig. 2 Recombinant protein production in the baculovirus–insect cell system

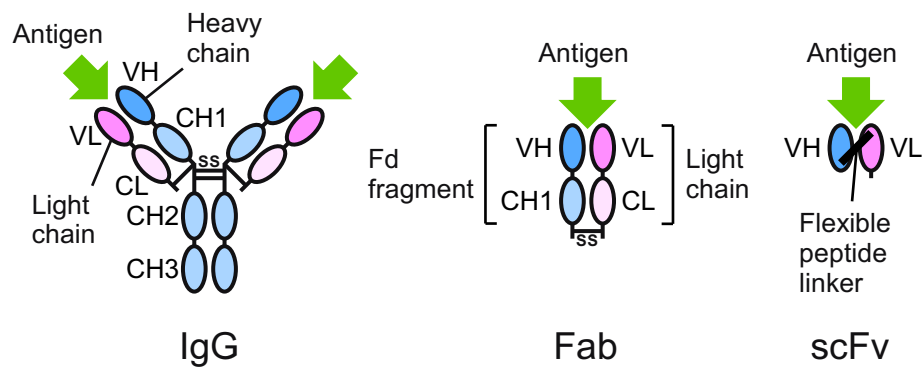


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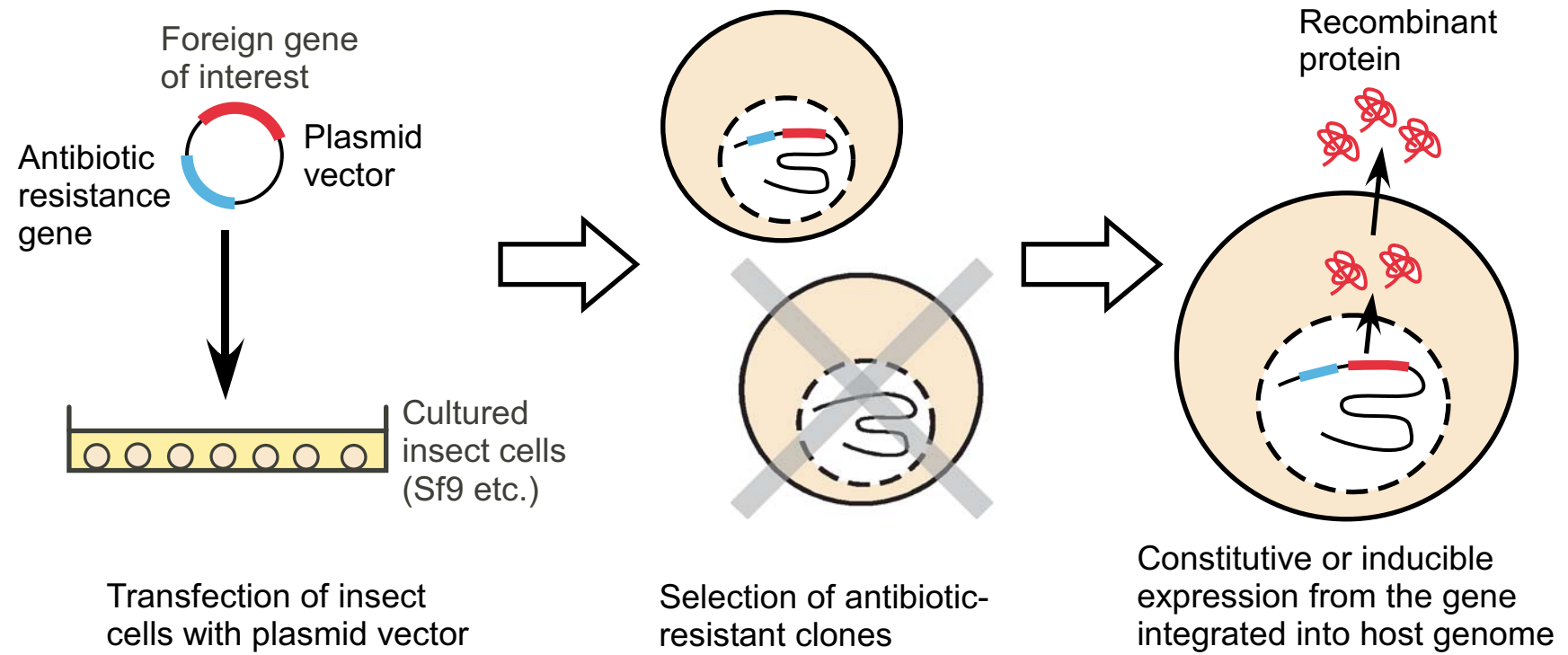


Fig. 4 Recombinant protein production by stably transformed insect cell lines

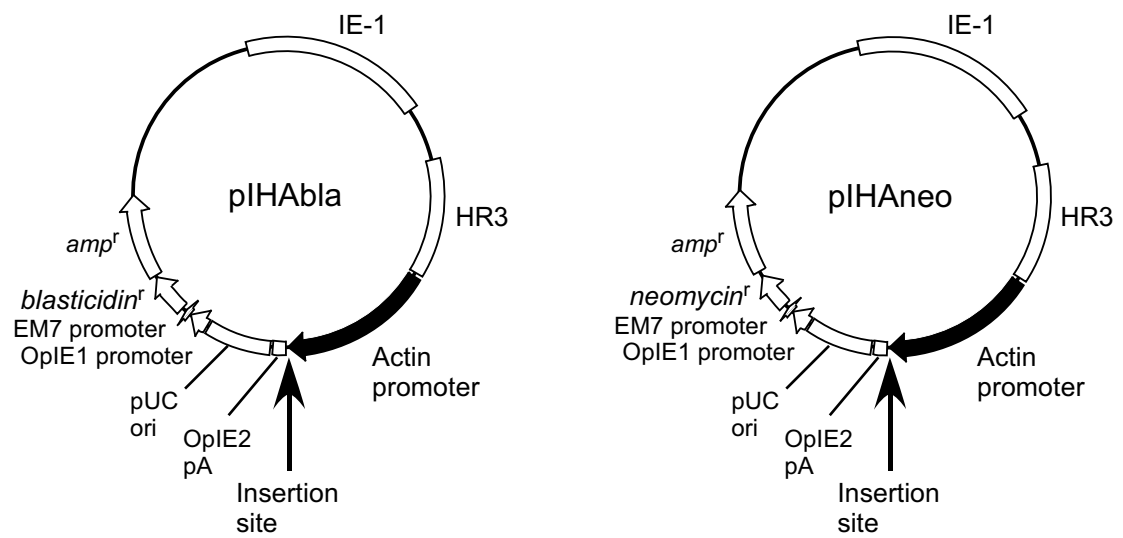


Fig. 4 Expression vectors for stable transformation of lepidopteran insect cells

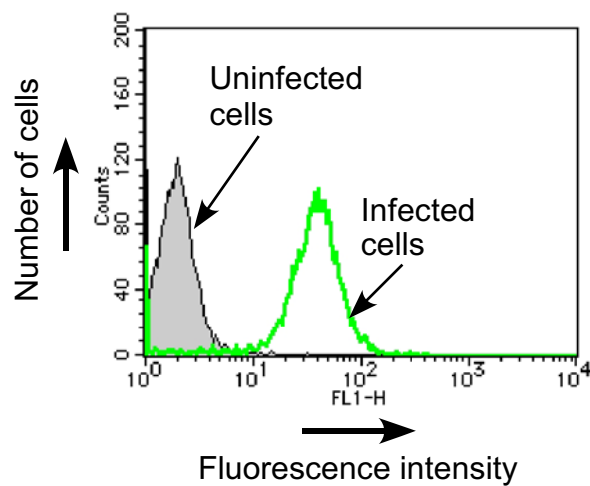


Fig. 5 Flow cytometric analysis of Sf9 cells infected with a recombinant baculovirus displaying mouse Fab fragments on its surface. Recombinant baculovirus carrying the genes of the Fd fragment and the light chain-gp64 fusion was used. Infected cells 24 h post-infection and uninfected cells were treated with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG before flow cytometry

Table 1 Production of recombinant antibodies in the baculovirus–insect cell system

Product	Cell line	Expression level	Note	Reference
Whole antibodies				
Mouse IgG1	Sf9	5 µg/ml	Two polyhedrin promoters, heavy and light chain signal peptides	Hasemann and Capra (1990)
Mouse IgG2a	<i>S. frugiperda</i>	25–30 µg/ml	Two polyhedrin promoters	zu Putlitz et al. (1990)
Mouse IgG2a	Sf9	25–30 µg/ml	Two polyhedrin promoters, heavy and light chain signal peptides	Nesbit et al. (1992)
Mouse/human chimeric IgG	Sf9	15–20 µg/(10 ⁶ cells)	Polyhedrin and p10 promoters, mouse VH signal peptide	Poul et al. (1995)
Human IgG1	Sf9	10 µg/ml	Polyhedrin and p10 promoters, mouse heavy and light chain signal peptides, spinner culture	Edelman et al. (1997)
Human IgG1	Sf9	9 µg/ml	Polyhedrin and p10 promoters, human heavy and light chain signal peptides	Liang et al. (1997)
Mouse/human chimeric IgG	Sf21 High Five	40 µg/ml (Sf21) > 70 µg/ml (High Five)	Polyhedrin and p10 promoters, honeybee melittin and <i>B. mori</i> larval serum protein signal peptides	Tan and Lam (1999)
Human IgG1	High Five	6–18 µg/ml	Baculovirus expression cassette vectors for cloning of heavy and light chain genes of Fab	Liang et al. (2001)

			or scFv, polyhedrin and p10 promoters, human heavy and light chain signal peptides	
Mouse IgG	Sf9	5–10 µg/ml	Bac-to-Bac baculovirus expression system, polyhedrin and p10 promoters, mouse heavy and light chain signal peptides, suspension culture	Song et al. (2010)
Fab fragments				
Mouse Fab	Sf9	10–20 µg/ml	Co-infection with two recombinant baculoviruses, polyhedrin promoter, honeybee melittin signal peptides, spinner flask culture	Abrams et al. (1994)
Mouse Fab	High Five	600 µg/ml	Bac-to-Bac baculovirus expression system, polyhedrin and p10 promoters, AcNPV gp64 signal peptide, shake-flask culture	Furuta et al. (2010)
scFv fragments and related proteins				
Mouse scFv	Sf9	15 µg/ml	Polyhedrin promoter, mouse light chain signal peptide	Laroche et al. (1991)
Human scFv	Sf9	32 µg/ml (purified scFv)	Polyhedrin promoter, AcNPV gp64 signal peptide, bioreactor culture	Kretzschmar et al. (1996)

Mouse scFv	Sf9	10 µg/ml (purified scFv)	p10 promoter, mouse heavy chain signal peptide, spinner culture	Lemeulle et al. (1998)
Mouse scFv	High Five	12 µg/(10 ⁶ cells) [10–15% of total cellular protein]	Polyhedrin promoter, honeybee melittin signal peptide, scFv was not secreted into medium	Yoshida et al. (1999)
Mouse scFv joined to human Fc γ 1 (hinge-CH2-CH3) and its fusion with hIL-2	Sf9	9 µg/ml (scFv-Fc) 3 µg/ml (scFv-Fc-IL-2)	Polyhedrin promoter	Bei et al. (1995)
Mouse scFv and its fusion with human IgG1 Fc (hinge-CH2-CH3)	Sf 158	0.2 µg/ml (scFv) 0.6 µg/ml (scFv-Fc)	Polyhedrin promoter, signal peptide from hIL-6 receptor	Brocks et al. (1997)
Mouse scFv fused with green fluorescent protein	Sf9	0.5–1 µg/ml	Bac-to-Bac baculovirus expression system, polyhedrin promoter, honeybee melittin signal peptide	Peipp et al. (2004)
Mouse scFv and bispecific scFv	High Five	—	Polyhedrin promoter, honeybee melittin signal peptide	Yoshida et al. (2003a)
Mouse bispecific scFv	Sf21	0.4–0.5 µg/ml (purified bispecific scFv)	Bac-to-Bac baculovirus expression system, polyhedrin promoter, honeybee melittin signal peptide	Bruenke et al. (2004)

Table 2 Production of recombinant antibodies in insect larvae and pupae

Product	Insect	Expression level	Note	Reference
Mouse IgG2a	<i>B. mori</i> larvae	800 µg/ml- hemolymph	Infection with recombinant BmNPV	Reis et al. (1992)
Human IgG1	<i>B. mori</i> larvae	46 µg/ml- hemolymph (36 µg/larvae)	Injection of recombinant cysteine protease- and chitinase- deficient BmNPV bacmid	Park et al. (2009)
Human IgG1	<i>B. mori</i> larvae and pupae	43 µg/ml- hemolymph (30 µg/larvae) 78 µg/pupae 239 µg/ml- hemolymph (co- expression of calreticulin)	Injection of recombinant cysteine protease- and chitinase- deficient BmNPV bacmid, co- expression of human molecular chaperone calreticulin	Dojma et al. (2010)
Mouse Fab	<i>T. ni</i> larvae	1.1 mg/g-larvae	Oral infection with recombinant AcNPV, automated insect rearing system	O'Connell et al. (2007)
Human scFv and green fluorescent protein-scFv fusion	<i>B. mori</i> larvae	188 µg/ml- hemolymph [132 µg/larva](scFv) 19 µg/ml- hemolymph (GFP-scFv)	Injection of recombinant cysteine protease- and chitinase- deficient BmNPV bacmid	Ishikiriya et al. (2009)

Mouse scFv	<i>B. mori</i> larvae	650 µg/ml- hemolymph (0.5 mg/larvae) [purified scFv]	Injection of recombinant BmNPV bacmid	Sakamoto et al. (2010)
Mouse IgG1	Transgenic <i>B.</i> <i>mori</i>	2.4 mg /g-cocoon (purified IgG) [1.1% in cocoon]	Secretion into sericin layer of silk fiber of cocoons	Iizuka et al. (2009)

Table 3 Production of recombinant antibodies in stably transformed insect cells

Product	Cell line	Expression level	Note	Reference
Humanized IgG1	S2	$\geq 1 \mu\text{g/ml}$	Co-transfection of heavy and light chain cDNA with a hygromycin resistance gene, <i>Drosophila</i> metallothionein promoter	Kirkpatrick et al. (1995)
Human IgG1	S2	5–35 $\mu\text{g/ml}$	Use of plasmid carrying both the heavy and light chain cDNA, <i>Drosophila</i> metallothionein promoter, <i>Drosophila</i> BiP signal peptide, shake-flask culture	Johansson et al. (2007)
Mouse scFv	S2	0.2–0.4 $\mu\text{g/ml}$	<i>Drosophila</i> metallothionein promoter, hHLA A2 signal peptide	Mahiouz et al. (1998)
Mouse scFv and its fusion with human κ chain	S2	25 $\mu\text{g/ml}$ (scFv) 20 $\mu\text{g/ml}$ (scFv- κ)	<i>Drosophila</i> metallothionein promoter, honeybee melittin signal peptide, T-flask culture	Reavy et al. (2000)
Mouse scFv	S2	200 $\mu\text{g/ml}$ (purified scFv)	<i>Drosophila</i> actin 5C promoter for constitutive expression, κ chain signal peptide	Gupta et al. (2001)
Human IgG1	High Five	0.06 $\mu\text{g/ml}$	Co-transfection of heavy and light chain genes with a neomycin	Guttieri et al. (2000)

			resistance gene, AcNPV hr5 enhancer and AcNPV immediate early promoter IE1, human heavy and light chain signal peptides	
Mouse IgG1 and its fusion with human TNF α or EGF	Sf9	0.5–1.0 μ g/ml	Co-transfection of heavy and light chain genes with a Zeocin resistance gene, OpNPV immediate early 2 promoter OpIE2, mouse heavy and light chain signal peptides	Li et al. (2001)
Mouse Fab	High Five	300 μ g/ml	Use of plasmid containing BmNPV IE-1 transactivator, BmNPV HR3 enhancer, and <i>B. mori</i> actin promoter with either a blasticidin or a neomycin resistance gene, <i>Drosophila</i> BiP signal peptide, shake-flask culture	Yamaji et al. (2008)
