Directional and direct cloning strategy for high-throughput generation of recombinant baculoviruses

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The baculovirus expression vector system (BEVS) has become one of the most widely used systems for routine protein expression. We have developed an improved strategy to clone foreign genes directionally and directly into the baculovirus genome vector via a one-step procedure to generate recombinant viruses in a week. In this work, we constructed a host strain Escherichia coli DH10BacHB1.1, which contains the modified baculovirus shuttle genome vector pHBMbacmid1.1 for the cloning vector. The treated PCR products of for expressed in the fluorescence protein and mannanase genes have been cloned in the baculovirus genome and foreign genes were ligated with the bacmid vector. The treated PCR products of foreign genes were ligated with the Bsu36I-digested vector. Then Spodoptera frugiperda (Sf9) cells were transfected directly with the ligation mixture. Using this method, the DsRed fluorescence protein and mannanase genes have been cloned in the baculovirus genome and expressed in the Sf9 cells. This strategy not only provides a means for high-throughput construction of recombinant baculoviruses, but also offers an idea of constructing other large plasmids and DNA virus-based expression vectors.

INTRODUCTION

The baculovirus expression vector system (BEVS) has become a popular and powerful tool for expressing a variety of recombinant proteins in insect cells (1–3). As compared with other expression systems, the BEVS has many advantages. First, the insect cells facilitate posttranslational modification, folding, and assembly of proteins. Secondly, high expression level can be achieved. Finally, insect cell lines are suitable for suspension cultivation in serum-free condition, which allows for the production of recombinant proteins in a large scale (3,4).

The prototype baculovirus Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) possessing a large circular double-stranded genome is widely used in BEVS for protein expression. In the past, the homologous recombination procedure was used for inserting foreign genes into the baculovirus genome (2). However, only 0.1% to approximately 1% of the positive recombinant baculoviruses is achieved using this method. To enhance the recombinant efficiency, several other approaches have been developed (5,6). For instance, 10% to approximately 25% recombinants can be achieved after the wild-type viral vector is linearized (7). Up to 99% recombinants can also be obtained if the ORF1629, downstream of the polyhedrin gene, is deleted completely or partially (8–10). Gristsun et al. (11) reported that foreign genes can be directly cloned into the viral genome by homologous recombination using appropriate primers.

An alternative approach to generate the recombinant baculovirus is the site-specific transposition with Tn7 transposase. Finally the recombinant baculovirus can be created by extraction of the bacmid DNA and transfection into Spodoptera frugiperda (Sf9) cells. Ernst et al. (13) and Lu and Miller (14) have further developed direct cloning methods for generating recombinant baculoviruses, which make it possible to construct a cDNA library using BEVS. They introduced I-SceI and Bsu36I sites into the baculovirus genome, respectively. Recently, Airenne et al. (15) presented an approach using the mutant SacB gene for promoting efficiency of selection based on the Bac-to-Bac® system (15). These methods greatly shortened the processes of obtaining recombinant baculoviruses and therefore simplified the selection procedures. With the development of BEVS, the modified baculovirus vectors carrying mammalian expression cassettes have been used for transient and stable gene delivery, not only in primary and established mammalian cells, but also in central nervous system and testes of mice (16–19).

In this work, we developed an improved direct cloning strategy to clone foreign genes directionally into the baculovirus genome and then generate recombinant viruses in a week. This is compatible with high-throughput expression technologies and is feasible in constructing a cDNA expression library. Using this method, DsRed gene and mannanase gene were cloned into the baculovirus genome and expressed in cells.

Table 1. Primers Used for Amplification and Mutagenesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>gfpF</td>
<td>5′-ACGGCGGCCGCGATTTATGTAAGACTGCGGCAAA-3′</td>
</tr>
<tr>
<td>gfpR</td>
<td>5′-CTGGCGGCGCGATTTATGTAAGACTGCGGCAAA-3′</td>
</tr>
<tr>
<td>mut1</td>
<td>5′-CCGCGAAGCTGACCTAAGGTTTCTTGGCAGACACCGG-3′</td>
</tr>
<tr>
<td>mut2</td>
<td>5′-CCGCGAAGCTGACCTAAGGTTTCTTGGCAGACACCGG-3′</td>
</tr>
<tr>
<td>DsRED</td>
<td>5′-TTAGGATCCACGGCCCGACAACAA-3′</td>
</tr>
<tr>
<td>DsREDR</td>
<td>5′-TTAGGATCCACGGCCCGACAACAA-3′</td>
</tr>
<tr>
<td>manF</td>
<td>5′-TTAGGATCCACGGCCCGACAACAA-3′</td>
</tr>
<tr>
<td>manR</td>
<td>5′-TTAGGATCCACGGCCCGACAACAA-3′</td>
</tr>
</tbody>
</table>

BsU36I restriction sites are in bold; CpoI and NotI sites are underlined; the extra nucleotides for each gene are boxed.
MATERIALS AND METHODS

Plasmids, Bacterial Strains, and Cell Cultures

pFastBac™1, Escherichia coli DH10Bac™, and DH10B™ were purchased from Invitrogen (Carlsbad, CA, USA). pEGFP-1 and pDsRED-2 are from Clontech (Mountain View, CA, USA), and TA cloning vector pMD18-T is from TaKaRa (Shiga, Japan). pPic-man vector containing a man gene (GenBank® accession no. AF324506) encoding mannanase was constructed and stocked in this laboratory. Insect cell line derived from the ovarian tissue of the fall army worm S. frugipera is maintained in Grace’s insect cell culture medium (Invitrogen) supplemented with 3.3 g yeast extract (Oxoid, Lenexa, KS, USA), 3.3 g/L enzymatic hydrolysate of lactalbumin (Difco™; BD, Franklin Lakes, NJ, USA), and 10% (v/v) fetal bovine serum (FBS; Invitrogen).

Plasmids Construction

pFGII. The gfp gene was amplified from pEGFP-1 with primers gfpF and gfpR (Table 1) by PCR. The PCR product was digested with CpoI and NotI and then inserted into the CpoI-NotI sites of pFastBac1 to obtain pFGII.

pT-gfp and pT-mgfp. The gfp gene amplified from pEGFP-1 with primers gfpF and gfpR was cloned into pMD18-T to obtain pT-gfp. A Bsu36I site 5′-CCTAAGG-3′ in the gfp gene was created by using QuikChange® Site-Directed Mutagenesis kit (Strategene, La Jolla, CA, USA) with the primers mut1 and mut2. The nucleotides 5′-GAA-3′ at the position 135–137 bp in the gfp gene were substituted with the 5′-AAG-3′. The mutated plasmid was designated as pT-mgfp, and the mutation was confirmed by DNA sequencing.

pFGIV. The mgfp gene fragment was excised from pT-mgfp by digestion with CpoI and NotI and cloned into CpoI-NotI sites of pFastBac1 to create pFGIV.

pHBMBacmid1.0. Transposition of pFGII was performed by transforming 100 µL DH10Bac competent cells with 100 ng pFGII. After incubation at 37°C for 4 h with vigorous shaking, the cells were plated on LB plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal), and 40 µg/mL isopropyl-β-d-thiogalactopyranoside (IPTG). The white colonies were selected for analysis after 48 h of incubation at 37°C. The plasmids extracted from the colonies were transformed into electrocompetent E. coli DH10B cells by electroporation (2.4 kV, 4 µΩ, 330 µF) using a Life Technologies Cell Porator Voltage Booster (Invitrogen) as described by the manufacturer. The transformants were grown on the LB plates containing 50 µg/mL kanamycin. The colonies were picked up and analyzed by gel electrophoresis. The positive transformants harbor only a large plasmid (approximately 130 kb) designated as pHBMBacmid1.0, and the resulting strain was called DH10BacHB1.0.

pHBMBacmid1.1. Using the same procedures for construction of pHBMBacmid1.0, the modified baculovirus genome vector, pHBMBacmid1.1, that contained a mgfp gene was constructed, and the resulting strain was called DH10BacHB1.1.

Cloning of man and DsRed Genes into pHBMBacmid1.0 and pHBMBacmid1.1 to Generate Recombinant Baculoviruses

Large amounts of pHBMBacmid1.0 and pHBMBacmid1.1 were prepared from 100 mL culture of DH10BacHB1.0 and DH10BacHB1.1 grown in LB medium supplemented with 50 µg/mL kanamycin. The cultures were harvested and lysed by the alkaline lysis method (20), and the plasmids were purified by High Purity Plasmid Purification System kit (Marligen Bioscience, Ijamsville, MD, USA). Ten micrograms viral DNA pHBMBacmid1.0 and pHBMBacmid1.1 were digested with 10 U Bsu36I (New England Biolabs, Ipswich, MA, USA) in 50 µL buffer at 37°C for 2 h, followed by ethanol precipitation, respectively. The man gene was amplified from pPic-man using the primers manF and manR. The DsRed gene was amplified from pDsRed2-1 using primers DsRedF and DsRedR. The amplified DNA fragments were incubated with 10 U T4 DNA polymerase and 5 µL 4 mM dGTP (both from TaKaRa) for 45 min at 12°C in 50 µL buffer. The treated fragments and Bsu36I-digested vectors were ligated with 70 U T4 DNA ligase in 10 µL buffer at 16°C for 24 h (Table 2). Ten microliters ligation mixtures were transfected into SF9 cells (2 × 10⁶ cells/mL) directly using Cellfectin® reagent (Invitrogen) as described by the manufacturer. The supernatants of the cultures containing recombinant baculoviruses were collected after 72 h of transfection and infected the fresh SF9 cells. The expression assays and viral plaque assay were performed at 72 h after infection.

Detection of Fluorescence and Mannanase Activity

At 72 h postinfection, the infected SF9 cells were examined and analyzed for expression of the fluorescence proteins using a Leica TCS-SP II confocal system (Leica, Heidelberg, Germany). The green fluorescence for green fluorescent protein (GFP) was detected by exciting a wavelength of 475 nm, and the red fluorescence for DsRed protein was examined by exciting 543 nm. Ten microliters supernatants of SF9 cell culture infected with the recombinant baculoviruses were blotted on the agar plate supplemented with 0.5% konjak mannan powder (stocked in this laboratory) and 0.02% trypan blue (Sigma) at 37°C for 10 h to assess expression of the mannanase (21). The supernatants of the cultures of SF9 cells infected with the parental viruses and the wild-type baculovirus were used as controls.

RESULTS AND DISCUSSION

The site-specific transposition with Tn7 has many advantages over the homologous recombination. However, the laborious steps of cloning of target genes into transfer vectors are hardly suitable for high-throughput gene cloning, and the efficiency of successful transposition in E. coli DH10Bac is only 8% to approximately 10% (12).
The methods of direct cloning provide a new means for construction of cDNA library, but they also require more DNA manipulations, such as ultracentrifugation isolation of the parental viral DNA, dephosphorylation of the viral vector, partial fill-in reaction of target genes, and removing of the background of the wild-type virus by multiple-round plaque purification steps (13,14). To improve the method of direct cloning and meet the needs of high-throughput technologies, we tried to develop a transfer vector-free directional and direct cloning strategy for the generation of recombinant baculoviruses.

The pFGII containing the gfp gene was first constructed (Figure 1A). Then pFGII was transformed into E. coli DH10Bac cells. The baculovirus genome vector pHBMBacmid1.0, transfer vector pFGIV, and helper plasmid were extracted from DH10Bac cells simultaneously and electroporated into E. coli DH10B cells. The positive colony harboring only one plasmid, the pHBMBacmid1.0, was selected by antibiotic selection and gel electrophoresis analysis. The resulting strain was named E. coli DH10BacHB1.0 (F Δ(ineC A Δ(mrr-r-hsdRMS-mcrBC)Φ 80lacZAM15 ΔlacX74 deoR recA endA araD139 Δ(ara, leu)7697 galU galK λ-Δ179 mgfp) pHBMBacmid1.0). pHBMBacmid1.0 was transfected into Sf9 cells, and the supernatant of the culture was collected and added into the fresh Sf9 cells. The transfection and the infection experiments indicated that GFP was expressed in Sf9 cells, and baculovirus Bac-gfp could be packaged in Sf9 cells (see Figure 3A).

To assess feasibility of pHBMBacmid1.0 used as viral DNA expression vector to express foreign genes in a rapid and efficient manner, two genes were cloned directly into the baculovirus vector. PCR products of DsRed and man genes treated with T4 DNA polymerase were ligated with pHBMBacmid1.0 digested by Bsu36I. The ligation mixtures were directly transfected into Sf9 cells (Figure 2B). Then, supernatants containing recombinant baculoviruses Bac-gfp-DsRed and Bac-gfp-man infected fresh Sf9 cells, respectively. At the 72 h postinfection, expression assays were performed. The results indicated that fluorescence of DsRed protein was expressed in the Sf9 cells (Figure 3D). The mannanase activity can be evaluated with the agar plate method (21). The supernatant of Sf9 cells infected with Bac-gfp-man made a clear halo on the plate (Figure 3H). Due to the presence of the gfp fragment in the ligation mixture and the possibility of recombination of the gfp gene with the linearized vector, nonrecombinant baculoviruses shown as green fluorescence still existed in the supernatants, which meant that plaque purification steps were necessary to remove the background (Figure 3, C and G).

In order to eliminate the nonrecombinant background, a mutant gfp gene, mgfp gene with a different Bsu36I site 5′-CCCTAGG-3′ (represented in bold), was obtained by site-directed mutagenesis and was cloned into pFastBac1 to create pFGIV (Figure 1B). Using a similar procedure as described previously, the updated baculovirus genome vector pHBMBacmid1.1 and E. coli strain DH10BacHB1.1 (F Δ(ineC A Δ(mrr-r-hsdRMS-mcrBC)Φ 80lacZAM15 ΔlacX74 deoR recA endA araD139 Δ(ara, leu)7697 galU galK λ-Δ179 mgfp) pHBMBacmid1.1) were constructed. The transfection and the infection experiments with pHBMBacmid1.1 demonstrated that the mutation of two nucleotides in the mgfp gene (K46R) does not affect GFP function, and the baculovirus Bac-mgfp can also be packaged normally in Sf9 cells (Figure 3B).

The cloning of DsRed and man genes into the linearized pHBMBacmid1.1 indicated that introduction of a different Bsu36I site in the gfp gene eliminated the background of parental baculovirus efficiently (Figure 3, E and I). At 72 h postinfection, titers of recombinant baculoviruses were 1.1 × 10^5 plaque-forming units (pfu)/mL and 0.9 × 10^5 pfu/mL, respectively. The efficiencies of recombinant baculoviruses were 3.3 × 10^5 pfu/μg viral DNA and 2.7 × 10^5 pfu/μg viral DNA, respectively (Table 2).

In our work, we developed an efficient cloning method for high-throughput and directional cloning of foreign genes into the baculovirus expression vector. The special pairs of restriction enzyme Cpol and NotI, ColI and AscI and Bsu36I, etc. can be considered as cloning sites. When the vector DNA is digested, two or three types of nucleotide overhangs form at the 5′ end. Since the recognition site of restriction enzyme Bsu36I does not exist in the wild-type baculovirus genome (14) and its special attribute

![Figure 1. Plasmid maps of (A) pFGII and (B) pFGIV.](image-url)
of recognition site (5'-CCTNAGG-3'; represented in bold), we have constructed the baculovirus genome vector, pHBMBacmid1.1 containing three unique Bsu36I sites. Complete digestion of pHBMBacmid1.1 with Bsu36I results in a linearized baculovirus DNA with overhang ends of 5'-TAA-3' and 5'-TCA-3', respectively. Correspondingly, four nucleotides of 5'-TTAC-3' and 5'-TGAC-3' were added to forward and reverse primer for amplification of target genes. As PCR products are incubated with T4 DNA polymerase and dGTP, the cohesive ends of 5'-TAA-3' and 5'-TCA-3' can form, which are compatible with the ends of the linearized pHBMBacmid1.1. Here we make use of 3' to 5' exonuclease activity and 5' to 3' polymerase activity of T4 DNA polymerase. When T4 DNA polymerase meets the first nucleotide G at 5' ends of the DNA in the presence of dGTP, the reaction will keep the balance between the exonuclease activity and polymerase activity (Figure 2B).

Our system combines the merits of two currently well-established approaches: (i) rapidity and simplicity of direct cloning of PCR products and (ii) convenience of the Bac-to-Bac system (Invitrogen). This expression cloning system has some other benefits in comparison with other high-throughput cloning methods based on PCR product, such as Gateway® technology (Invitrogen). First, it requires only one step for cloning and does not need any transfer or entry vector. Secondly, the primers of this method were shorter than those of direct cloning using I-ScI (9) and Gateway technology. Additionally, restriction enzyme Bsu36I and T4 DNA polymerase used in this method are much cheaper than BP Clonase™ II and LR Clonase II (both from Invitrogen). Therefore, this method will be more economic for large-scale cloning expression. At present, we have constructed several kinds of strains and baculovirus vectors containing His-tag, glutathione-S-transferase (GST)-tag, and FLAG®-tag to meet the needs of protein purification. Meanwhile some baculovirus vectors harboring the mammalian protein expression cassette were also constructed for gene delivery or protein-protein interaction researches (data not shown). Moreover, the idea of a three-Bsu36I site had been applied to other large-scale cloning expression studies in this laboratory, such as a yeast two-hybrid system, a mammalian two-hybrid system, and a Bombyx mori-baculovirus expression system.

Figure 2. Overview of (A) the construction of host strain Escherichia coli DH10BacHB1.1 and (B) the procedures of generation of recombinant baculoviruses. The PCR product of a foreign gene is treated by T4 DNA polymerase and dGTP, and then is ligated with the Bsu36I-digested pHBMBacmid1.1. Finally the ligation mixture is transfected directly into Spodoptera frugiperda (Sf9) cells, and the recombinant baculovirus is obtained at 3 days of posttransfection.

Figure 3. Fluorescence protein expression in Spodoptera frugiperda (Sf9) cells at 72 h postinfection of (A) baculovirus Bac-gfp, (B) Bac-mgfp, (D) recombinant baculoviruses Bac-gfp-DsRed, and (F) Bac-mgfp-DsRed. Mannanase activity assay. The supernatants of the cultures of Sf9 cells infected by the recombinant baculoviruses were blotted on the agar plate containing 0.5% konjac mannan powder to assess the expression of mannanase. Mannanase activity was detected as clear halos on the plate (H and J). The different supernatants contain the different baculoviruses: (panel H, 1 and 2) Bac-gfp-man, (panel J, 1 and 4) Bac-mgfp-man, (panel H, 5 and 6) Bac-gfp-DsRed, (panel J, 3 and 5) Bac-mgfp-DsRed, the parental viruses (panel H, 3) Bac-gfp and (panel J, 2 and 6) Bac-mgfp, and (panel H, 4 and panel J, 7) the wild-type baculovirus. The parental viruses and wild-type baculovirus were used as controls. As the parental virus DNA, pHBMBacmid1.0 still had the nonrecombinant background (C and G), while the background of pHBMBacmid1.1 was not detected (E and I).
Here we described an improved strategy for directional cloning of foreign genes into the baculovirus expression vector and generation of recombinant baculoviruses in a week. On the base of direct cloning methods, we constructed a host strain E. coli DH10BacHB1.1, which contains the baculovirus shuttle vector pHBM Bacmid 1.1. Although only two genes were cloned into the baculovirus genome, this strategy is compatible with high-throughput cloning researches and will offer an idea for other large plasmids or DNA virus-based expression vectors.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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