A new, rapid and simple procedure for direct cloning of PCR products into baculoviruses

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ABSTRACT

We propose a novel method for direct cloning of foreign genes into baculoviruses which avoids the use of bacterial transfer vectors. The foreign gene to be inserted is derived by PCR using appropriate primers each of which contains an additional 50 nt of baculovirus sequence for homologous recombination between the PCR-derived DNA and the baculovirus DNA, thus accomplishing insertion of the foreign gene into the baculovirus. The direct cloning of green fluorescent protein and β-glucuronidase in different baculovirus loci is described. The method is simple and avoids the use of cumbersome techniques associated with enzymatic treatment and DNA purification.

Recombinant baculovirus expression systems are among the most commonly used for expressing foreign genes in eukaryotic cells (1). These systems provide relatively high yields of expressed recombinant proteins that are similar to authentic proteins in their biological activity. Currently there have been considerable advances in development of the recombinant baculovirus systems extending the range of their application (2–4).

Conventionally, the foreign gene is inserted in a baculovirus expression vector through the bacterial transfer vector which contains two ‘recombinant arms’ (RA) ie long baculovirus sequences ∼300–3000 nt which provide classical homologous recombination between transfer vector and baculovirus during cotransfection (1). In some cases this conventional approach is impossible because of incompatibility of some eukaryotic sequences with conventional bacterial systems used for their cloning. One of the methods which avoids bacterial cloning is based on the direct ligation of foreign DNA into a specific restriction site within the baculovirus genome (5,6). Another approach uses ligation between the transfer vector and the foreign DNA, followed by cotransfection of the purified ligation complex and the baculovirus DNA (7). Here we present a novel alternative approach to introduce foreign genes into baculoviruses without an intermediate bacterial stage. The method, which is based on the principal of homologous recombination, utilises PCR primers which contain only 50 nt of RA representing the site where recombination is required. The method is simple, rapid and reliable.

To demonstrate the efficiency of this method we have expressed the green fluorescent protein (GFP) gene under the polyhedrin promoter of the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV). The strategy of these experiments is depicted in Figure 1. As a baculovirus vector we used AcRP23-LacZ, containing β-galactosidase under control of the polyhedrin promoter which provides white–blue selection of recombinant virus. A unique restriction site (Bsa36I) in the β-galactosidase gene was used to linearise baculovirus DNA (6). GFP is readily detectable in cells using fluorescence microscopy (9). Plasmid pGFP (CLONTECH Laboratories, Inc.) was used as a template for PCR. The upstream and downstream primers for PCR each contained 50 nt of baculovirus polyhedrin locus sequence (10), followed by either 19 or 21 nt from the 5’ or 3’ ends respectively of the GFP gene. The baculovirus homologous region of the upstream primer sequence commenced 4 nt downstream of the transcription start signal, corresponding to nt 4473–4522 of the baculovirus genome (10) as indicated below: 5’-TATTTTACGTGGTCGTGATACATTTGTATATAAAAAA-AACCTATATAATGGCTAGCAAAAGGAGAAGAAC-3’ (baculovirus sequence is emboldened). The baculovirus homologous region of the downstream primer sequence commenced 50 nt downstream of the β-galactosidase gene stop codon, corresponding to nt 5258–5308 of the baculovirus genome (10) as indicated below: 5’-GCACAGAATCCAGCCGATTAATAAATTGCTGATCTAATAAATGTTACGTGCTTTTTATTTGTATAGTTCTACGAA-G-3’ (baculovirus sequence is emboldened). The PCR programme included three cycles at 95°C for 40 s, 52°C for 1 min and 72°C for 1 min, followed by 27 cycles at 95°C for 40 s and 72°C for 1 min. The derived PCR product consisted of 860 bp and the expressed product of this sequence was a 238 amino acid GFP, which replaced the β-galactosidase expressed under control of the polyhedrin promoter (Fig. 1). The derived PCR DNA was separated from non-specific DNA by electrophoresis in 1% low-melting point agarose gel. The DNA was extracted from the excised agarose by freezing at −20°C in a microcentrifuge tube. The thawed agarose was then centrifuged at 14 000 r.p.m. for 5 min. The PCR-derived DNA from the clarified supernatant medium was precipitated using 9 vol butanol, washed in 70% ethanol and then dissolved in TE buffer at pH 8.0.

For the transfection experiments, Petri dishes (35 mm) were seeded with 1–1.5 × 10⁶ Sf21 cells and incubated at 28°C for 2 h to allow cells to attach. One hundred ng of Bsa36I-digested DNA of A. californica nuclear polyhedrosis virus (AcRP23-LacZ) (8) was mixed with 500 ng of PCR DNA of GFP and adjusted to a volume of 50 µl. Then, 50 µl of 100 µg/ml Lipofectin™ (GIBCO
BRL/Life Technologies) was gently added dropwise. The mixture was incubated for 20 min at room temperature and then added dropwise to dishes essentially as described previously (1).

After incubation for 3 days, the supernatant medium from transfection experiments was analysed for virus by plaque assay (1) in the presence of 40 μg/ml X-gal. Observation by fluorescence microscopy showed that expression of GFP occurs from the third day after infection. In two independent experiments, ~16% of the plaques formed were blue. Among the 84% of white plaques, ~20% produced green fluorescence. For comparison, using the GFP gene cloned in a transfer vector, we obtained 60% of fluorescent plaques. These results show that the efficiency of transfection using the transfer vector was higher than that obtained by direct cloning of PCR product, nevertheless, we believe the direct cloning method has distinct advantages, which can be outlined as follows. (i) The direct cloning method overcomes the problem encountered when the PCR product is toxic for bacterial cells or contains sequences that produce gene reorganization through bacterial DNA repair mechanisms and therefore cannot be cloned in a transfer vector. (ii) The existing methods of direct insertion of foreign genes in baculovirus, avoiding the bacterial stage of cloning (5–7), are cumbersome, require a lot of manipulation with DNA and strongly depend for efficiency on the skill of the scientist. Our method is simple and rapid, does not utilise restriction enzymes and requires only PCR DNA purification from agarose gel. (iii) Screening of genes non-essential for baculovirus replication in cell culture [e.g. polyhedrin, p10 (12), p26, p74, EGT (4), etc.] can be achieved by substitution of the baculovirus gene with a marker gene expressed under the control of the same promoter, thus providing positive selection. This information can be used to study mechanisms of gene regulation within the baculovirus genome. Secondly, it can be used to create new loci for insertion of foreign genes in the baculovirus genome without preliminary linearization of baculovirus DNA.

This has been demonstrated by cloning the β-glucuronidase gene under the p10 promoter of BacPac6 baculovirus (11). The primers for PCR corresponding to nt 118788–118838 and 119124–119174, respectively, of the baculovirus genome (10) were as follows. Forward: TTTGTTAATTAAATTTACATCAATGTGAAATATT-ACATTATTTACAATCCCTCGAGAAAATGGTCCGTC-CTGTAGAA; reverse: TCGTACGAATATTAAAAACATT-GAATTGTATTTTTAAGCCGTCATCGGCCTGAGG-AGCCCAAGCGG (baculovirus sequences in bold, new unique restriction site Sse8387 I in italics). Using this strategy we identified 12 positive blue plaques among ~10⁶ negative white plaques in the presence of 100 μg/ml X-β-D-glc, (BIOSYNTH AG). Blue plaques were purified and the expression of β-glucuronidase was demonstrated by SDS-PAGE (data not shown). Both upstream and downstream primers were designed to contain a unique restriction site (Sse8387 I) which made it possible to linearise the new baculovirus vector and therefore significantly increase the percentage of recombinant viruses produced (3,8).

In summary, cotransfection of baculovirus DNA with PCR products containing sequences homologous to selected regions of the baculovirus genome, enables targeted insertion of foreign genes into the baculovirus genome. The data demonstrate that a 50 nt sequence of baculovirus DNA sited at each end of the foreign sequence is sufficient to provide homologous recombination. Our approach should increase the ease with which the baculovirus genome can be modified. It should also expand the scope of study of baculovirus gene function without the need for either new transfer vectors or cumbersome procedures to introduce unique restriction sites for linearisation.

REFERENCES