1991

Use of the polymerase chain reaction for screening and evaluation of recombinant baculovirus clones

A. C. Webb

M. K. Bradley

Shelley A. Phelan
Fairfield University, sphelan@fairfield.edu

J. Q. Wu

L. Gehrke

Peer Reviewed

Published Citation

Webb, A. C., Bradley, M. K., Phelan, S. A., Wu, J. Q., & Gehrke, L. (1991). Use of the polymerase chain reaction for screening and evaluation of recombinant baculovirus clones. BioTechniques, 11(4), 512-519.

Use of the Polymerase Chain Reaction for Screening and Evaluation of Recombinant Baculovirus Clones

A.C. Webb, M.K. Bradley[1,2], S.A. Phelan, J.Q. Wu[2] and L. Gehrke[2,3]

Wellesley College, [1]Dana Farber Cancer Institute and the Harvard Medical School, [2]Harvard-M.I.T. Div. of Health Sciences and Technology, Massachusetts Institute of Technology, and [3]Harvard Medical School

ABSTRACT

We report the application of the PCR for screening and high-resolution characterization of recombinant baculovirus clones. Starting with less than 10 nanograms of viral DNA, it is possible to 1) demonstrate that the DNA sequence to be expressed has not been deleted or rearranged during the co-transfection or homologous recombination events, 2) test for the presence of wild-type virus in the isolate and 3) generate amplified DNA that can be used for nucleotide sequence analysis or high-resolution restriction analysis. The method is based upon PCR of genomic viral DNA prepared from primary amplified stocks of extracellular virus using a small-scale procedure. The approach has special relevance for definitive characterization of recombinant virus used to express point mutant proteins and for characterization of recombinant virus generated through use of mixed oligonucleotides or random mutagenesis.

INTRODUCTION

Protein production using the baculovirus expression vector system (BEVS) offers large yields (10–500 mg/l of culture) of proteins that are biologically active, stable and posttranslationally modified (7,12,13). Use of BEVS has been facilitated by the introduction of improved transfer vectors (7,8,16) and commercial availability of defined cell culture media and a BEVS expression kit. With increased utility of the expression system, it is now possible for a laboratory to express multiple proteins with BEVS and to consider expressing a series of mutant proteins for structure/function analyses. Such an approach requires technology for precise characterization of the recombinant virus.

In this paper, we present a method that utilizes the PCR (3,11) for screening and evaluation of recombinant baculovirus. Although the majority of recombinant viruses that were analyzed during the course of this work carried intact DNA inserts, we also identified recombinants from which a portion of the DNA insert had been deleted during the cotransfection or homologous recombination steps. PCR was used to identify these defective isolates prior to time-consuming plaque purifications. The PCR protocol does not require the use of radioisotopes, and the required oligonucleotides are often available in the laboratory in the form of DNA sequencing primers. The technique is extremely sensitive and can be used both to determine the purity of an isolate and to generate DNA for direct nucleotide sequence analysis.

MATERIALS AND METHODS

Insect Cells

The methods for growth and transfection of *Spodoptera frugiperda* (Sf9) insect cell line by baculovirus genomic and transfer vector DNAs have been described by Summers and Smith (14) and Bradley (2). Sf9 cells were seeded at a density of 5×10^5 cells per ml of Ex-Cell 400 medium (JRH Biosciences, Lenexa, KS) containing the surfactant pluronic F-68 (Fluka Chemical, Ronkonkoma, NY) at a final concentration of 0.1% (9) and 1% (v/v) antibiotics (penicillin-streptomycin; GIBCO BRL/Life Technologies, Gaithersburg, MD). The cells were maintained in 30 ml medium in 150-ml capped Erlenmeyer flasks that were rotated at 55 rpm on an orbital shaker (Bellco Glass, Vineland, NJ) inside a 27°C incubator (Lab-Line Ambi Hi-Lo [Lab-Line Instruments, Melrose Park, IL]). When the cell density reached 2×10^6 per ml, the cells were sedimented by a brief centrifugation at about 1000 rpm in a clinical centrifuge and resuspended in fresh medium at 5×10^5 cells/ml before transfer to a clean flask.

Vectors and Transfections

The non-fusion transfer vectors pVL941, pVL1392 and pVL1393 (6–8,16) were used for this work. Complementary DNA sequences were inserted at the unique *Bam*HI site of pVL941 or within the polycloning region of pVL1392/1393 using standard recombinant DNA methodology.

Autographa californica nuclear polyhedrosis virus (AcNPV) genomic DNA for use in co-transfection reactions was prepared from extracellular virus particles using the method described by Summers and Smith (14), except that the sucrose gradient centrifugation step was omitted.

Although the serum-free Ex-Cell medium was used for all other parts of the protocol, we found that calcium phosphate co-transfection reactions using Ex-Cell were accompanied by

the formation of enormous precipitates that led to high levels of cell death. For this reason, transfections were performed according to methods described by Summers and Smith (14) using Sf9 insect cells grown in TNM-FH medium (JRH Biosciences, Lenexa, KS) containing serum. Supernatants harvested after transfection were diluted in Ex-Cell 400 medium and used to infect the Ex-Cell 400-conditioned cells prior to overlay with 3% SeaPlaque agarose (FMC BioProducts, Rockland, ME) diluted 1:1 with 2× Ex-Cell 400 containing 2% (v/v) antibiotic solution.

Microscopic Visualization of Recombinant Viral Plaques

Viral plaques were examined for the presence (occ+) or absence (occ-) of polyhedra by incident illumination of inverted 60-mm Lux plates (NUNC, Naperville, IL) on a black-base background using Wild M5A binocular dissecting microscope (Wild-Leitz USA, Rockleigh, NJ) equipped with fiber optics (Intralux 5000 [American Volpi, Auburn, NY]) at 120–500× magnification. Plates incubated at 27°C for 5–7 days were rapidly scanned at the lower magnification, and plaques were circled on the base of the plate with a felt-tipped marker prior to closer inspection at higher magnification.

Preparation of Viral DNA for PCR

Plaques were excised from plates by aspirating agarose plugs into a sterile Pasteur pipet (Figure 1). The plugs were expelled into 1 ml of Ex-Cell 400 medium in a sterile microcentrifuge tube, and virus was allowed to elute from the plug for at least 4 h at room temperature or overnight at 4°C. Primary stocks of virus were then established by inoculating 1×10^6 Sf9 cells in 5 ml of Ex-Cell 400 medium in a 25-cm^2 tissue culture flask (Corning, Corning, NY) with 0.2 ml of agarose-plug eluate, followed by incubation at 27°C for 6–7 days. When more than 50% of the monolayer showed cytopathic effects of destruction from virus infection and lysis, the culture medium was harvested and cleared of cellular debris by centrifugation at 2000× *g* in a low-speed centrifuge prior to DNA preparation.

The protocol for preparation of AcNPV DNA for PCR amplification is essentially a scaled-down modification of the method described by Summers and Smith (14). Viral particles from 2 ml of the culture supernatant fluid were pelleted by centrifugation at 100 000× *g* for 30 min (32 000 rpm in an SW50.1 rotor (Beckman Instruments, Palo Alto, CA) with the culture supernatant diluted to volume with Ex-Cell 400 medium. The tubes were inverted and allowed to drain for 2–3 min before removal of residual medium from the sides of the tube with a Kimwipe. Virus pellets were resuspended by repeated pipetting in 0.2 ml of 0.1 M Tris-HCl (pH 7.5), 0.1 M EDTA, 0.2 M KCl, and the suspension was transferred to a fresh 1.5-ml microcentrifuge tube. Twenty micrograms of freshly prepared proteinase K were added, and the virus suspension was incubated in a heat-block at 50°C for 2 h. Sodium Sarkosyl™ was added to a final concentration of 1%, and incubation continued for at least an additional 2 h (or overnight, if convenient). The digests were deproteinized twice by extraction with an equal volume of a 1:1 mixture of buffer-saturated phenol containing chloroform:isoamyl alcohol (24:1).

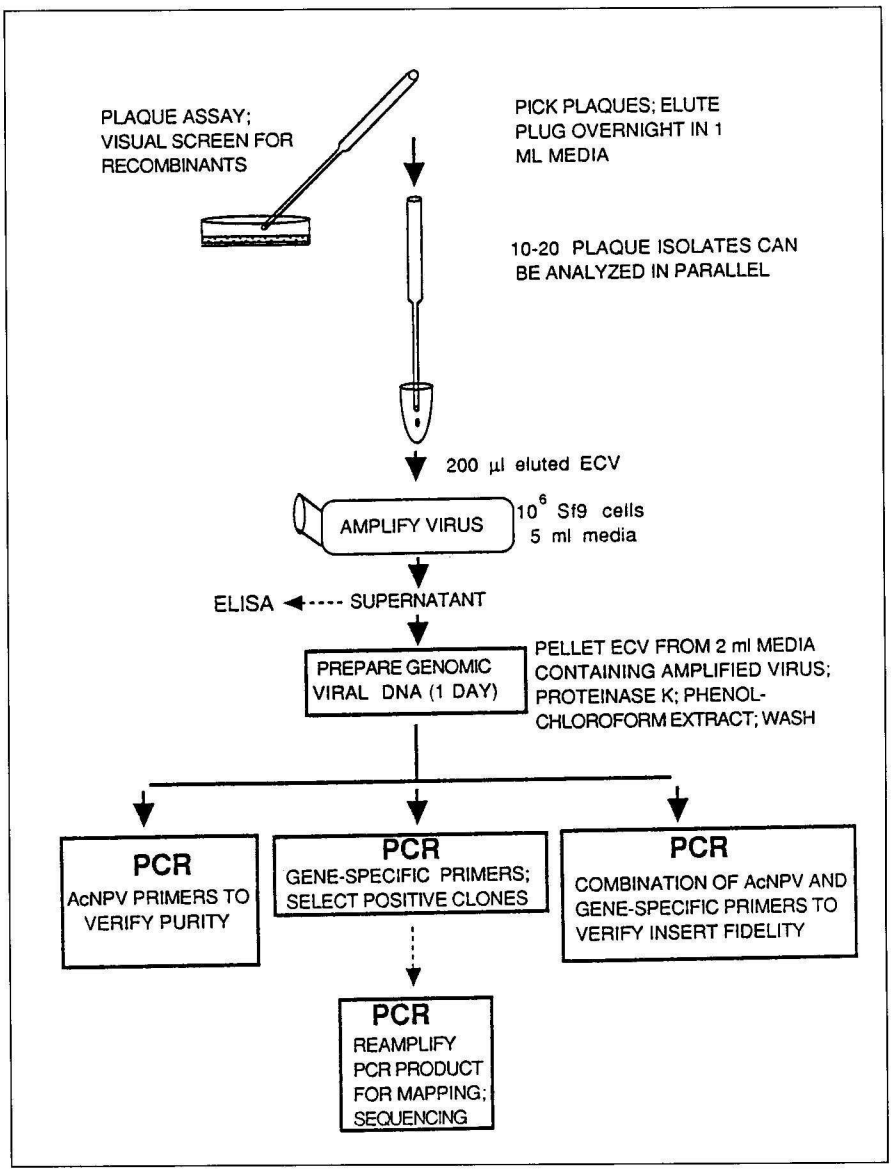


Figure 1. Flow diagram illustrating use of PCR for screening of recombinant baculovirus clones. Abbreviations: AcNPV: *Autographa californica* nuclear polyhedrosis virus (baculovirus); ECV: extracellular virus.

During these organic extractions, tubes were subjected to vigorous vortexing, and no particular care was exercised to prevent shearing of the viral genomic DNA. Aqueous and organic phases were separated by centrifugation for 3 min in a microcentrifuge at room temperature. Because AcNPV DNA precipitated by ethanol solubilizes very slowly, we used a filter centrifugation method to remove residual organics and to concentrate the DNA before PCR amplification. Filter units (Millipore UFC3 LGC 00 [Millipore Products Div., Bedford, MA]) were prewashed to minimize adsorption of DNA by adding 200 µl of phosphate-buffered saline (PBS) and centrifuging the unit at 2000× *g* (position #5 on a Model 5415 Microfuge; Brinkmann Instruments, Westbury, NY) for 30 min. The aqueous phase from the phenol extraction (ca. 200 µl) was then added to the filter unit without removal of residual PBS, and the volume was diluted to 400 µl (the maximum for the filter unit) with water. Three rounds of centrifugation/dilution were completed before final removal of the washed and concentrated DNA from the filter unit in a volume of 50–75 µl. The concentration of genomic DNA ranged from 20–200 µg/ml. Although absorbance ratios for the DNA were somewhat less than ideal (A_{260}/A_{280} = 1.5–1.8), no problems were experienced amplifying the DNA with *Taq* DNA Polymerase (Perkin-Elmer Cetus, Norwalk, CT).

PCRs

PCR amplification of 10 ng of viral DNA was performed in a 100-µl reaction containing 1 µg of each primer using AmpliTaq® kit reagents and apparatus (Perkin-Elmer Cetus, Norwalk, CT) under the following temperature cycling protocol: 3 min at 94°C × 1 cycle; 0.5 min at 94°C, 1 min at 50°C, 1 min at 72°C × 25 cycles; 5 min at 72°C × 1 cycle.

Routinely, 16-20 µl of these reactions were analyzed by electrophoresis into horizontal agarose TBE gels containing ethidium bromide. Primers were synthesized using conventional phosphoramidite chemistry on either an Applied Biosystems PCRmate (Foster City, CA) or a MilliGen 7500 oligonucleotide synthesizer (MilliGen/Biosearch, Div. of Millipore, Burlington, MA).

Detection of Protein Expression by ELISA

Proteins expressed by cells infected with recombinant virus were detected in the primary virus stock supernatant fluid using enzyme-linked immunosorbent assay (ELISA) techniques. Aliquots were added directly to microtiter wells of commercial ELISA kits or, alternatively, pipetted onto nitrocellulose filters for dot-blot analysis. For the dot-blot ELISA, nitrocellulose membrane was first wet with binding buffer B (4) and assembled into a dot-blot apparatus (Schleicher and Schuell, Keene, NH). Excess buffer was cleared by applying vacuum. The vacuum was then interrupted, and 100 µl of binding buffer B, followed by 2, 5 or 10 µl of primary virus solution, were pipetted into the wells. Vacuum was then applied, and the membrane was washed by adding 200 µl of binding buffer B. The membrane was removed from the apparatus and incubated in blocking buffer (3%–10% bovine serum albumin) for 30–60 min at 37°C. After draining the excess blocking buffer, primary antibody diluted in buffer A (50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$) was added, followed by overnight incubation at 4°C. In most cases, we absorbed antibodies or antisera against lyophilized Sf9 cells to minimize nonspecific reaction (2). Following the overnight incubation, the filter was rinsed with wash buffer W (1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.14 M NaCl, 3 mM KCl, 0.05% Tween 20, 0.02% sodium azide), and the second antibody/alkaline phosphatase conjugate was added. Incubation with gentle agitation continued for 1–2 h at room temperature. The nitrocellulose membrane was then washed three times with buffer WS (wash buffer W containing 1 M NaCl) and once with wash buffer W before the addition of a color substrate such as Fast Red and naphthol AS-BI phosphate (Sigma Chemical, St. Louis, MO). Color development was stopped by rinsing the membrane with water. Negative controls included supernatant fluids from AcNPV-infected (occ+) cells or supernatant fluids from cultures infected with an unrelated recombinant virus.

RESULTS AND DISCUSSION

A flow diagram illustrating use of PCR for screening of recombinant baculovirus clones is shown in Figure 1.

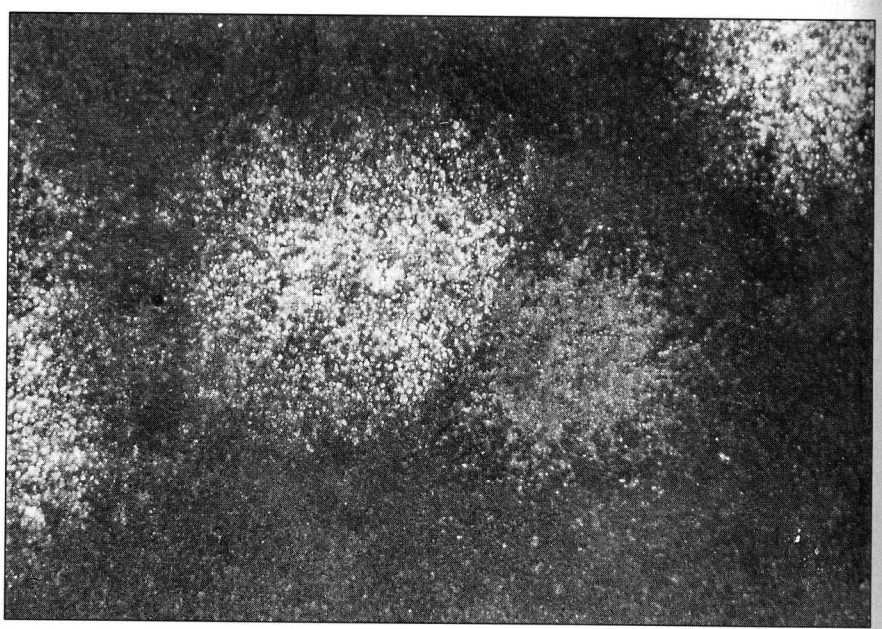


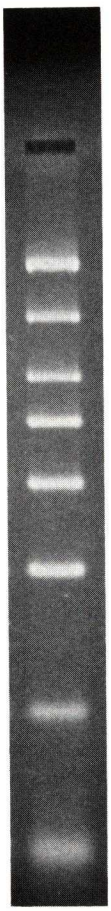
Figure 2. Microscopic appearance of recombinant (occ-) and wild-type (occ+) viral plaques under incident, fiber optic illumination through the base of Lux plate containing Sf9 cells overlaid with agarose. Note the intense refractile or fluorescent appearance of the polyhedra-containing cells (occ+) infected with nonrecombinant virus compared to the adjacent plaque of occlusion-negative (occ-) cells infected with recombinant virus. (Magnification 125×)

ACCURATELY SIZE YOUR PCR† PRODUCTS

BioMarker™ LOW

BioMarkers are a new family of dsDNA electrophoresis markers designed to provide the researcher with:

- Eight bands which correspond exactly to base pairs:
- Accurate determination of product size
- Estimation of product yields
- Equal band intensities
- No ambiguous bands
- No protocol changes
- Ready to use
- Photogenic
- Now with exposed 5'OH for kinasing
- Available with nonradioactive labels
- Stable at room temperature
- Additional size coverage
- Next day delivery



Photograph: 5μl (1 Assay) application to 12 cm 4% agarose gel, 3:1 Nusieve® GTG, Agarose. Gel was run at 100 volts in .5xTBE and stained for 30 minutes with ethidium bromide at 0.5μg/ml.

TO RECEIVE

FREE SAMPLE

MARK READER SERVICE NUMBER

BioVentures, Inc.

• 848 Scott Street • Murfreesboro, TN 37129

BioMarker is a trademark of BioVentures, Inc. and patent pending
†PCR is covered by U.S. Patents issued to Cetus Corporation
NuSieve is a registered trademark of FMC Corp.

The PCR amplification method described here generates DNA substrate sufficient for several diagnostic assays including insert size analysis, nucleotide sequence analysis and assessment of the purity of the isolate. Nonrecombinant (occ+) DNA was found not to interfere with amplification of recombinant (occ-) DNA directed by insert-specific primers; therefore, putative clones can be verified prior to time-consuming plaque purifications. The availability of large amounts of amplified DNA substrate for high-resolution analyses distinguishes the PCR method from other screening methods, and the protocol can be used as an alternate approach or in concert with established BEVS screening techniques (14).

The visual selection of recombinant plaques is arguably the most difficult step in the cloning procedure, and a representative example of the morphological characteristics of nonrecombinant (occ+) and recombinant (occ-) plaques is presented in Figure 2. Cells containing polyhedra (occ+) appeared almost fluorescent, whereas plaques consisting of cells infected with recombinant virus (occ-) were markedly less refractile.

Our initial attempts at developing a rapid screening method focused upon amplifying viral DNA eluted from the agarose plug in a manner similar to that described for bacteriophage lambda (10). To test the applicability of the phage technique, aliquots of plug eluates were added directly to PCRs. We determined immediately, however, that the eluate solution contained a potent inhibitor of *Taq* polymerase (data not shown). We have not identified the inhibitor, but since the agarose apparently does not affect PCR (1), inhibition is likely caused by a component of the Ex-Cell 400 medium. The inhibitor effect was diminished by dilution, but amplified DNA was never detected in PCRs containing the diluted eluate solution. These results suggest that either the quantity of template was insufficient for amplification or the eluted viral DNA was completely packaged and therefore not a substrate for *Taq* polymerase. We did not pursue identification of the inhibitor further because DNA prepared by a small-scale modification of the Summers and Smith protocol (14) was completely suitable for PCR amplification. This technique consistently yields more than 1 µg of amplifiable DNA from 2 ml of a 6–7-day culture supernatant.

Detection of recombinant (occ-) virus by PCR is based upon amplification of the specific insert sequences which replace the polyhedrin gene during recombination. A schematic representation of the constructs used to test the PCR method is shown in Figure 3. To demonstrate that both nonrecombinant (occ+) and recombinant (occ-) sequences could be amplified and detected in the same PCR, AcNPV viral DNA and recombinant transfer vector DNAs were mixed in varying proportions prior to amplification. A 1200-bp cDNA was subcloned into transfer vector pVL1392, and a constant amount of this DNA (10 ng) was mixed with AcNPV viral (occ+) DNA in ratios of 1:1 to 1:0.0001 before PCR, using flanking primers specific for baculovirus sequences (BV1/BV2; Figure 3) found in both DNAs. The expected size of the fragment amplified from AcNPV viral (occ+) DNA is 385 bp, while that amplified from the recombinant transfer vector is 1400 bp in length (Figure 3). (Note that 140 bp were deleted from the polyhedrin sequence during construction of the transfer vector [8]; therefore, the size of the fragment amplified from recombinant viral DNA is slightly less than the sum of amplified viral DNA fragment [385 bp] plus the size of the cDNA insert [1200 bp].) Analysis of the amplified DNA fragments by agarose gel electrophoresis is presented in Figure 4A, and three points are illustrated by these data. First, the presence of two bands in lanes 3–6 demonstrates that both the nonrecombinant (occ+) DNA (385 bp) and cDNA insert sequences (1400 bp) were amplified in the same PCR. Second, the results suggest that detection of the recombinant transfer vector sequence using flanking AcNPV primers (BV1/BV2) is dependent on at least a 10-fold excess of recombinant (occ-) DNA over nonrecombinant (occ+) DNA. (For example, the 1400-bp fragment was not detected in a reaction containing 1:1 mixtures of the DNAs [lane 2]; however, we demonstrate later that if primers specific for the insert DNA are

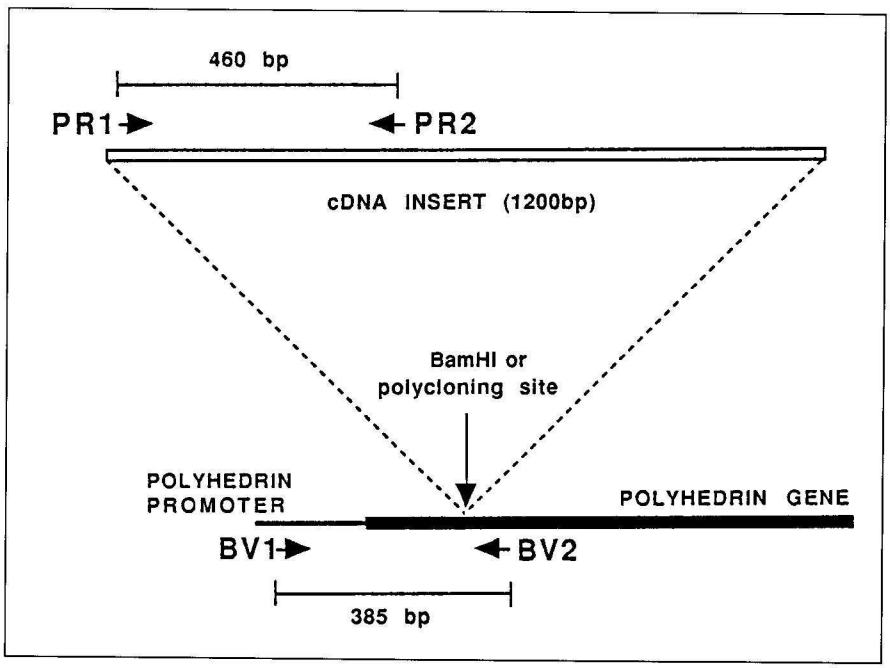


Figure 3. Schematic representation of DNA constructs showing the site of insertion of cDNA sequences into the transfer vector/viral genome and the positions of primers used for PCR amplification. The nucleotide sequence of primers PR1 and PR2 is dependent upon the sequence inserted for protein expression. The nucleotide sequences of the baculovirus-specific primers flanking the site of DNA insertion (14) are:

BV1 (-168 to -144): 5′ ATAACAGCCATTGTAATGAGACGCA 3′
BV2 (217 to 193): 5′ TGAAGAGAGTGAGTTTTTGGTTCTT 3′

used, efficient amplification is independent of the relative concentrations of occ+ and occ- DNAs [Figure 6].) Third, since the smaller (385 bp) nonrecombinant (occ+) viral DNA PCR product is, as expected, amplified more rapidly than the larger recombinant, extremely small amounts (<1 pg; 0.01%) of nonrecombinant (occ+) virus contamination can be detected (Figure 4A, lane 6). This provides a simple and highly sensitive method of determining the purity of recombinant clones.

We note here a technical consideration, involving the order of addition of the primers and other reagents to the PCRs, which appears to be important for the successful detection of recombinant virus genomic DNA. The PCRs analyzed in Figure 4(A and B) were prepared in the same manner except that for Figure 4A, the primers were added last and immediately prior to initiating the reaction, whereas for Figure 4B, the primers were added to a "master mixture" and the DNA templates were added last. Although there appeared to be no obvious complementarity between the baculovirus primers used (others tested gave similar results), the preparation of a "master mixture" for PCR containing both primers consistently resulted in failed amplification (compare corresponding lanes in Figure 4, A and B). There was also a huge decrease in the level of sensitivity for detection of nonrecombinant (occ+) DNA (compare the 385-bp fragments in Figure A, lanes 4–6, with the same lanes in Figure 4B).

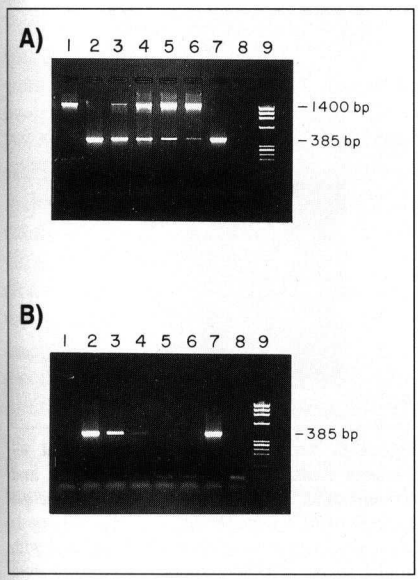


Figure 4. Amplification of recombinant DNA sequences in the presence of wild-type virus DNA. *(A) Agarose gel analysis of amplified DNAs.* PCRs were prepared as described in Materials and Methods. The PCR primers BV1 and BV2 were added last to the reactions, immediately before placing the tubes in the thermal cycler. The reactions analyzed in lanes 1–7 represent amplification of DNA fragments from mixtures of recombinant and nonrecombinant templates. The ratios of recombinant/wild-type DNA (ng/100 µl) are: lane 1, 10/0; lane 2, 10/10; lane 3, 10/1; lane 4, 10/0.1; lane 5, 10/0.01; lane 6, 10/0.001; lane 7, 0/10. Lane 8: without DNA template. Size standards (ϕX174 DNA digested with *Hae*III) are shown in lane 9. The sizes of the DNA standards, in order of increasing electrophoretic mobility are 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp. *(B) Effect of adding PCR primers to a "master mixture."* The reactions analyzed in this figure were prepared exactly as for those in part A, with the exception that the primers BV1 and BV2 were added to a "master mixture" containing buffer, nucleotides and *Taq* polymerase before subsequent addition of DNA templates.

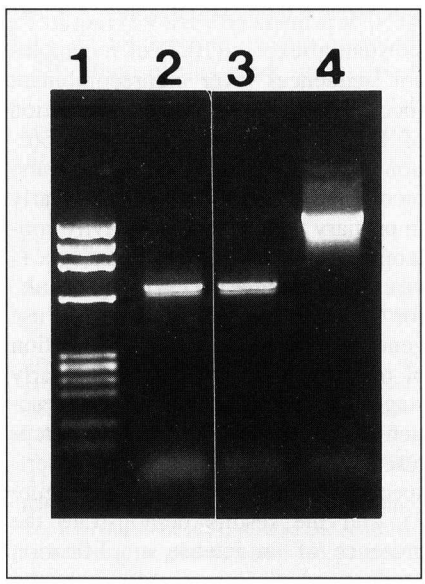


Figure 5. Detection by PCR of recombinant virus carrying inserts from which DNA was deleted during co-transfection or homologous recombination. A 1450-bp cDNA insert was subcloned into transfer vector pVL1392 and co-transfected with genomic DNA into Sf9 cells. Occ- plaques were identified visually, and virus eluted from agarose plugs was used to infect a plate of cells. Extracellular viral DNA was used as template in PCRs primed by specific oligonucleotides which annealed to the extreme 5′ and 3′ termini of the insert DNA; therefore, a 1450-bp amplified band was expected for virus carrying full-length inserts. An aliquot of each of the amplification reactions was analyzed on a 1.5% agarose gel. Lane 1, size standards (ϕX174 DNA digested with *Hae*III); lane 2, DNA amplified using clone 2C as template; lane 3, DNA amplified using clone 2H as template; lane 4, DNA amplified using the corresponding transfer vector as template.

Although the majority of recombinant viruses that were analyzed during the course of this work contained full-length inserts, the PCR protocol revealed a partial deletion of one of our insert DNAs. A 1450-bp DNA insert was subcloned into transfer vector 1392 (16) before co-transfection with AcNPV genomic DNA into Sf9 cells. Several plaques were identified visually as occlusion-negative, suggesting that homologous recombination between genomic and transfer vector DNAs had occurred. Virus eluted from plug eluates was used to infect a 5-ml culture of Sf9 cells, and DNA was isolated from extracellular virus. Specific primers which annealed at the extreme 5′ and 3′ termini of the insert DNA were used for PCR with the expectation of amplifying a 1450-bp fragment from recombinant virus carrying a full-length insert. The data presented in Figure 5 demonstrate that truncated inserts were detected in independent isolates (lanes 2 and 3) while amplification of corresponding transfer vector DNA yielded the expected 1450-bp fragment (lane 4). Cause and absolute frequency of this type of deletion are not known, but it is clear that significant effort was saved by identifying the deletion before the virus was purified through several rounds of time-consuming plaque isolations. In this case, the plaque assay was repeated using the same transfection mixture, and we were successful in isolating recombinant virus carrying full-length inserts that expressed the correct protein.

Virus collected from a first plaque purification inevitably contains a mixture of both nonrecombinant (occ+) and recombinant (occ-) virus. The data presented in Figure 4 are evidence that PCR amplification can nonetheless be used for characterization of the insert DNA during initial stages of purification. The results shown in Figure 6A emphasize, however, the value of using insert-specific primers rather than AcNPV flanking primers when the isolate contains significant amounts of occ+ (wild-type) DNA. Virus from a first plaque isolate was eluted and amplified as described in Materials and Methods, and DNA was isolated by the

small-scale procedure. Ten nanograms of purified DNA were amplified using either the flanking baculovirus primers BV1/BV2 or cDNA-specific primers PR1/PR2 (see Figure 3). The electrophoretic pattern of amplified DNAs (Figure 6A) demonstrates the presence of recombinant sequences, as evidenced by the 1400-bp amplified product in lane 2 comigrating with DNA amplified from pure recombinant transfer vector DNA containing the same sequence (lane 5). Nonrecombinant (occ+) DNA is also clearly evident (lane 2, 385 bp). Based upon these results, it is clear that although contaminated with nonrecombinant (occ+) virus, the isolate contains recombinant virus. At the same time, however, the data presented in Figure 4 demonstrated that detection of recombinant (occ-) sequences using the flanking AcNPV primers (BV1/BV2) requires a substantial excess (10:1) of recombinant sequences over nonrecombinant (occ+) DNA. Given the consideration of PCRs containing varying proportions of nonrecombinant (occ+) and recombinant (occ-) DNAs (particularly in primary plaque isolates heavily contaminated with nonrecombinant [occ+] virus that produce an excessive "sink" for the primers), it is important to use gene-specific primers for the detection of recombinant virus during the early stages of plaque purification. The reaction analyzed in Figure 6A, lane 3, represents amplification using insert-specific primers PR1/PR2 (see Figure 3), and the results demonstrate the presence of an intense amplification product which comigrates with the control material (lane 4). Note that although the full-length amplification product is relatively weak (lane 2, 1400 bp), the smaller cDNA-specific fragment is amplified very efficiently (lane 3). Therefore, insert-specific primers (i.e., PR1, PR2) should be used for screening mixed DNA populations, while the baculovirus primers (BV1/BV2) are reserved for assessment of purity in plaque-purified clones.

Recombinant baculovirus clones are generated by homologous recombination between genomic DNA and the smaller transfer vector DNA (14). Our data (Figure 5) indicate that DNA deletions, while relatively infrequent, can occur during the process of creating recombinants. Since recombinant virus carrying a defective insert and screened by visual identification or dot-blot hybridization can be phenotypically indistinguishable from a functional recombinant virus, it is possible to expend significant effort in the purification of a virus that has no utility for

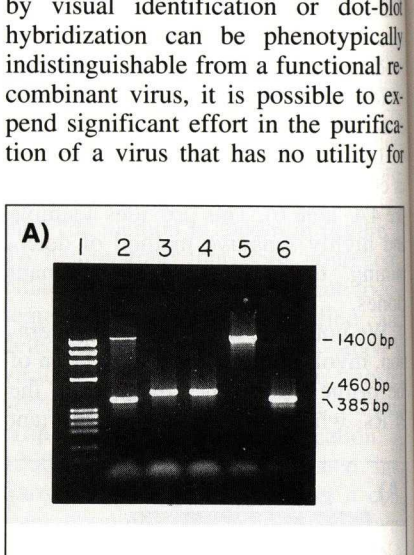


Figure 6. Amplification of recombinant sequences from a mixture of wild-type and recombinant DNA templates. *(A) Agarose gel analysis of PCR amplification products.* Lane 1: DNA size standards, ϕX174 DNA digested with *Hae*III. Lane 2: DNA products amplified from primary plaque isolate DNA template using flanking baculovirus primers BV1 and BV2; lane 3: DNA products amplified from primary plaque isolate DNA template directed by insert-specific primers PR1 and PR2; lane 4: control reaction representing amplification of insert sequences using primers PR1 and PR2 and purified transfer vector DNA containing the cDNA insert; lane 5: control reaction representing amplification using flanking primers BV1 and BV2 and purified transfer vector DNA containing the cDNA insert; lane 6: control reaction representing amplification of sequences present in nonrecombinant genomic baculovirus DNA directed by flanking primers BV1 and BV2. *(B) Use of pairs of internal and flanking primers for demonstrating that a DNA insert is intact.* Genomic viral DNA was prepared from a pure virus isolate containing the 1200-bp cDNA insert (Figure 3), and PCRs were directed by the following primer pairs: lane 1, PR1/BV2 (expected size 1200 bp); lane 2, PR1/PR2 (expected size 460 bp); lane 3, BV1/PR2 (expected size 660 bp); lane 4, BV1/BV2 (expected size 1400 bp); lane 5, DNA standards, *Hae*III digest of ϕX174 DNA.

protein expression. Although Southern blot analysis reflects the size of the DNA insert, PCR technology can be used to rapidly demonstrate the integrity of a putative recombinant (Figure 5B) with further utility for generating DNA substrate for high-resolution techniques. It is important to note that a single preparation of viral DNA is sufficient for multiple amplifications, thus providing substrate for repeated analyses or for a number of different analyses. The precision of the characterization of inserted DNA can also be increased by amplifying small internal fragments directly by combinations of insert-specific and baculovirus-flanking primers. An example of such an analysis is presented in Figure 6B. Various combinations of baculovirus-specific and insert-specific primers were used to amplify DNA isolated from a purified plaque. The amplified DNAs from each of the reactions are exactly the size predicted (see Figure 3), lending strong support for the intact nature of the insert in the recombinant virus. If desired, the fidelity of the cloned gene sequence can be further verified by either restriction enzyme mapping or direct sequencing of the recombinant PCR product (1,5,15). An additional advantage of virus amplification for the preparation of DNA for PCR is that expressed protein released from lysed cells into the amplified viral supernatants (T-25 flasks, Figure 1) can be detected by ELISA prior to final purification of the virus clone.

Clearly, the final functional test of a recombinant baculovirus is the expression of a biologically active protein. The PCR protocol alone, described in this paper, cannot guarantee expression or functional activity, but it can provide a definitive analysis of multiple clones of recombinant virus. From the data generated, isolates can be selected with certainty that the virus is indeed recombinant and carries the *bona fide* sequence required for productive expression. Combination of PCR and ELISA from the primary virus preparation provides a powerful coupling of assays which can be used to demonstrate, at a very early stage of purification, that the isolate contains the precise full-length DNA sequence and that the recombinant virus is active in protein expression.

Note added in proof: After this paper was accepted for publication, Sewall also described the use of PCR for evaluation of recombinant baculovirus clones (Sewall, A. 1991. The Digest from Invitrogen *4*:2).

ACKNOWLEDGMENTS

We gratefully acknowledge the generosity of Dr. Max Summers and his colleagues for supplying us with the materials to establish the baculovirus expression system in our laboratories. We also thank Beth Weiner, Lester Lien and Carl Connui for providing expert advice and for many useful discussions. This work was supported by grants from the National Institutes of Health (GM42504, AI27850, CA-038069, BRSG 2 S07RR07047-23 and BRSG S07RR05526-26), Whitaker Health Sciences Fund and a Brachman-Hoffman Student Fellowship awarded to SP. Much of this work was carried out while A.C.W was on sabbatical leave from and supported by Wellesley College.

REFERENCES

1. **Belyavsky, A.** 1989. Polymerase chain reaction in the presence of NuSieve GTG agarose. Resolutions, FMC Newsletter *5*:1-2.
2. **Bradley, M.K.** 1990. Overexpression of proteins in eukaryotes. Methods Enzymol. *182*:112-132.
3. **Erlich, H.H., D.H. Gelfand and R.K. Saiki.** 1988. Specific DNA amplification. Nature *331*:461-462.
4. **Kilton, L.J., M.K. Bradley, C. Mehta and D.M. Livingston.** 1981. A rapid and sensitive quantitative immunoassay for the SV40 large T antigen. J. Virol. *38*:612-620.
5. **Kretz, K.A., G.S. Carson and J.S. O'Brien.** 1989. Direct sequencing from low-melt agarose with Sequenase. Nucleic Acids Res. *17*:5864.
6. **Luckow, V.A. and M.D. Summers.** 1988. Signals important for high level expression of foreign genes in *Autographa californica* nuclear polyhedrosis virus expression vectors. Virology *167*:56-71.
7. **Luckow, V.A. and M.D. Summers.** 1988. Trends in the development of baculovirus expression vectors. Bio/Technology *6*:47-55.
8. **Luckow, V.A. and M.D. Summers.** 1989. High level expression of nonfused foreign genes with *Autographa californica* nuclear polyhedrosis virus expression vectors. Virology *170*:31-39.
9. **Murhammer, D.W. and C.F. Goochee.** 1988. Scaleup of insect cell cultures: Protective effects of pluronic F-68. Bio/Technology *6*: 1411-1417.
10. **Nishikawa, B.K., D.M. Fowlkes and B.K. Kay.** 1989. Convenient uses of polymerase chain reaction in analyzing recombinant cDNA clones. BioTechniques *7*:730-733.
11. **Oste, C.** 1988. Polymerase chain reaction. BioTechniques *6*:162-167.
12. **Smith, G.E., M.J. Fraser and M.D. Summers.** 1983. Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: deletion mutations within the polyhedrin gene. J. Virol. *46*:584-593.
13. **Smith, G.E., M.D. Summers and M.J. Fraser.** 1983. Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol. Cell. Biol. *3*:2156-2165.
14. **Summers, M.D. and G.E. Smith.** 1988. A manual of methods for baculovirus vectors and insect cell culture procedures. Tex. A & M Univ. Agric. Exp. Sta. Bull. No. 1555:1-57.
15. **Thein, S.L.** 1989. A simplified method of direct sequencing of PCR amplified DNA with Sequenase T7 DNA polymerase. Editorial Comments, United States Biochemical Corporation *16*:8.
16. **Webb, N.R. and M.D. Summers.** 1990. Expression of proteins using recombinant baculoviruses. Technique *2*:173-188.

Address correspondence to:

Lee Gehrke
M.I.T. E25-545
Cambridge, MA 02139
LGEHRKE@EAGLE.MIT.EDU