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Construction of Recombinant Vaccinia Viruses Using PUV-Inactivated Virus as a Helper

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ABSTRACT

Recombinant vaccinia viruses (VVs) are widely used as expression vectors in molecular biology and immunology and are now under evaluation for gene therapy. The current techniques for inserting foreign DNA into the large VV genome are based on either homologous recombination between transfer plasmids and VV genomes or direct DNA ligation and packaging using replication-deficient poxviruses. Here, we describe efficient new versions of both methods that produce 90%–100% of the recombinant viruses. In the new homologous recombination method, VV DNA “arms” obtained by *NotI* digestion and intact transfer plasmids were used for co-transfection. In the direct DNA ligation method, foreign DNA was inserted into a unique *NotI* restriction site of the VV genome. In both methods, the generation of recombinant viruses was carried out in cells infected with a non-replicating, psoralen-UV (PUV)-inactivated helper VV. The convenience of these new techniques is demonstrated by the construction of recombinant VVs that produce *E. coli* β -galactosidase. An important feature of these strategies is that any VV strain can be used as a helper virus after PUV inactivation.

INTRODUCTION

Vaccinia virus (VV), a member of the *Poxviridae*, has a large, complex virus particle and a dsDNA genome of about 200 kb. Several advantages of VV as a vector have made it a popular choice for gene delivery in vaccine studies (11) and therapeutic applications (5,17). For example, VV has a large capacity for cloning foreign genes and infecting a broad range of animal and human cells.

The classical technique for inserting foreign DNA into the large VV genome is based on homologous recombination between transfer plasmids and VV

genomes (3,8,13). However, this method is labor intensive because of the low frequency of recombination and high background of parental viruses contaminating the virus plaques. Therefore, this method requires multiple cycles of purification of the recombinant viruses. Alternatively, direct DNA ligation can be used to construct recombinant VV using special helper poxvirus strains for the recovery of viable recombinant virus (9,10,12,15). Although this method is effective and allows the cloning of inserts at least 26 kb in length (10), it has not been widely used because of the inconveniences associated with growing helper viruses and their low yield.

In this report, we demonstrate that VV inactivated with psoralen and long-wave UV irradiation (PUV-VV) can be effectively used as a helper virus for the recovery of recombinant viruses generated either by direct DNA ligation or by homologous recombination between co-transfected transfer plasmids and viral DNA arms. Both new methods are equally efficient and allow the generation of recombinant VV with almost no background of parental viruses.

MATERIALS AND METHODS

Cells and Viruses

CV-1 cells (ATCC, Rockville, MD, USA) were grown in DMEM (Sigma, St. Louis, MO, USA) containing 5% FBS and antibiotic-antimycotic solution (Sigma). The Lister (LIVP) strain of VV was used in these experiments as a parental virus. VV was propagated in CV-1 cells and purified through a sucrose gradient as previously described (16).

Plasmid Constructs

For the construction of pNT8, the *NotI* region coding sequence of VV was amplified by PCR using forward primer 5'-GGGAATTCTTATACATC-CTGTTCTATC-3', reverse primer 5'-CCAAGCTTATGAGGAGTATTGCGGGGCTAC-3', and VV DNA as a template. The resulting 972-bp fragment contained flanking *EcoRI* and *HindIII* restriction sites at the 5' and 3' ends, respectively. The PCR product was cleaved with *HindIII* and *EcoRI*

and was inserted into the pUC28 (1), resulting in pNT7. A 54-bp polylinker with multiple cloning sites was inserted into the *NotI* site of pNT7, generating pNT8, which was used as a vector for the cloning of foreign genes.

The *BamHI-SmaI* fragment (3293 bp) of pSC65 containing the pE/L promoter was isolated by digestion with restriction enzymes, blunted with Klenow enzyme, and cloned into the *SmaI* site of the pNT8, resulting in pNZ2. The pNZ2 contains the cDNA of *E. coli lacZ* under the control of the VV early/late promoter p7.5 and a synthetic early/late VV pE/L promoter derived from pSC65 plasmid (4). The pNZ2 was used for the construction of the following recombinant VVs: rVV-Not-LZ1, rVV-Not-LZ2, and rVV-Not-PZ.

DNA Ligation

Purified VV DNA (10 μ g) was incubated overnight at 37°C in a 30- μ L reaction mixture containing 40 U *NotI*. Digested DNA was purified by phenol-chloroform extraction and treated with 2 U alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN, USA) at 37°C for 50 min. The reaction mixture was then heated at 65°C for 10 min, and the DNA was purified by phenol-chloroform extraction and dissolved in 15 μ L distilled water. The *NotI*-digested viral DNA (1 μ g) was ligated with 3 μ g of the 3357-bp *NotI* fragment of pNZ2, containing the p7.5-driven *lacZ* gene, in a 20- μ L reaction mixture with 20 U T4 DNA ligase (New England Biolabs, Beverly, MA, USA) by being incubated overnight at room temperature. The ligation mixture was used for the transfection experiment.

Generation of PUV-VV

VV (3×10^8 pfu/mL) was incubated in a 35-mm plate with 1 μ g/mL psoralen (4'-aminomethyl-trioxsalen; Calbiochem-Novabiochem, San Diego, CA, USA) in a total volume of 1 mL Hanks buffer containing 0.1% BSA at room temperature for 10 min. The plate was irradiated for 6–8 min in a Stratalinker® 1800 UV Crosslinker (Stratagene, La Jolla, CA, USA) equipped with five 365-nm, long-wave UV bulbs as previously described (17). The PUV-

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VV was assayed for remaining viable virus particles by standard plaque assay (3). The PUV-VV that did not induce cytopathic effects on CV-1 cells within three days after infection was used for the experiments.

Generation of Recombinant VVs

CV-1 cells grown in 60-mm dishes

were infected with PUV-VV at an MOI of one. Two hours after infection, the cells were transfected with either the previously described ligation mixture or with 2 µg of the mixture of *NotI*-digested viral DNA and 6 µg intact plasmid DNA. The lipid-mediated transfection of cells was carried out using 5 µL GenePORTER™ reagent (Gene Therapy Systems, San Diego, CA, USA) per

1 µg DNA, according to manufacturer's instructions. The cells were incubated in the transfection mixture for 4 h and then supplemented with a medium containing 20% FBS. Cytopathic effects were monitored daily by light microscopy. The cells were incubated for 5–8 days, until virus plaques formed and the cytopathic effect was complete. The infected cells were then harvested, resuspended in 0.5 mL medium, and frozen and thawed three times to release the virus. Single virus plaques were selected for the preparation of small and large recombinant virus stocks and analyzed for the *lacZ* gene insertion and expression using Southern blot (17) and plaque assay (3) involving blue color production after using the substrate for β-galactosidase X-Gal (Molecular Probes, Eugene, OR, USA), respectively.

RESULTS AND DISCUSSION

We described two alternative new protocols for the construction of recombinant VVs. The first method is based on the ligation of a foreign DNA fragment with the VV DNA arms produced by *NotI* digestion, followed by transfection of CV-1 cells with this ligation mixture. The second method is based on homologous recombination in transfected cells between the VV DNA arms produced by *NotI* digestion and intact transfer plasmids containing a foreign gene expression cassette flanked with VV sequences. In both methods, before transfection, cells were infected with a PUV-VV, which served as a helper for the initiation of the replication cycle for the recombinant virus. The unique *NotI* site in VV is located in the *HindIII* F region and is nonessential for virus replication (6). The presence of the unique *NotI* restriction site in the VV genome of strain Lister was confirmed by pulsed field electrophoresis of *NotI*-digested viral DNA (data not shown).

Plasmid pNZ2 was used to test both protocols for the efficient generation of recombinant VV (Figure 1). The first method is based entirely on the ligation of DNA fragments and generation of recombinant genomes in vitro, followed by the transfection of CV-1 cells

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previously infected with PUV-VV (Figure 1A). Genomic DNA of recombinant viruses (designated rVV-Not-LZ1 and rVV-Not-LZ2) contained inserts in two alternative orientations as shown in Figure 1A. The second method, for which DNA ligation is not required, is based on intracellular recombination between homologous VV sequences within the intact plasmid (a derivative of pNZ2) and viral DNA cleaved at a unique *NotI* site (Figure 1B). Recombinant virus designated rVV-Not-PZ was obtained using this method.

By both methods, the expression cassette of foreign DNA was inserted precisely at the *NotI* site as predicted. The integration of the *lacZ* gene into the VV genome was confirmed by Southern blot hybridization (Figure 2, panels A and B). The purity of recombinant VV plaques was analyzed by plaque assay. Using our transfection-recombination method, approximately 90% of the virus plaques acquired blue color, while about 10% of the plaques remained color-free (Figure 2, panels C

Table 1. Efficiency of Foreign Gene Insertion into VV Genome

Method of Transfection	No. of Recombinant (R) and Wild-Type (wt) Virus Plaques						Recombinant Viruses (%) mean $\pm$ sd
	Exp. 1		Exp. 2		Exp. 3		
	R	wt	R	wt	R	wt	
A	90	0	54	1	85	0	99.3 $\pm$ 1.15
B	158	6	80	20	140	0	92.2 $\pm$ 10.74
C	24	>500	15	>500	32	>500	<0.18

A, in vitro ligation method; B, new homologous recombination method; and C, conventional method. Each experiment was repeated at least three times.

and D). To confirm the purity of the obtained recombinant clones, individual blue plaques were isolated, and viruses were propagated and then titered again in the agar medium with X-Gal. All of the generated virus plaques were blue, indicating that they contained pure, homogeneous recombinant VV.

Both strategies produced an almost background-free yield of recombinant viruses. The efficiency of recombinant

virus production was evaluated and compared by plaque assay in three methods using the same transfer plasmid pNZ2 (Table 1). In addition to the direct ligation-transfection (A) and co-transfection-recombination (B) protocols, the traditional homologous recombination method (C) was also performed. The traditional method involved cell infection with a live VV, followed by transfection with the transfer

Figure 1. Strategies for recombinant VV production. (A) DNA ligation method. Wild-type VV (strain Lister) DNA digested at the unique *NotI* restriction site (DNA arms) was treated with alkaline phosphatase and ligated with the *NotI* fragment from the pNZ2 plasmid. The ligation mixture was transfected into PUV-VV-infected CV-1 cells for recombinant VV generation. Recombinants rVV-Not-LZ1 and rVV-Not-LZ2 with the *lacZ* gene expression cassette in opposite orientation were obtained. (B) New homologous recombination method. PUV-VV-infected cells were transfected with VV DNA arms and intact pNZ2 to produce the rVV-Not-PZ. L and R cubes show VV sequences involved in homologous recombination. Although the sequence analysis has not been performed, we anticipate that the DNA structures of resultant rVV-Not-LZ1 and rVV-Not-PZ recombinant viruses are also identical. Vector pNT8 was used for the construction of plasmid DNA pNZ2.

Figure 2. Gene insertion and expression analyses. (A and B) Southern blot analysis. Recombinant VV DNAs were digested with *NotI*, electrophoresed on a 0.8% agarose gel, and analyzed using a 3357-bp DNA probe of pNZ2. Data show that recombinant viruses produced by the DNA ligation method (A) or by the new homologous recombination method (B) contain *lacZ* gene inserted at the *NotI* site. Lane 1, positive control, *NotI* fragment (3357 bp) of plasmid pNZ2; lane 2, DNA from CV-1 cells infected with wild-type VV; (A) lanes 3–12 and (B) lanes 5–12, DNAs from the cells infected with viruses from single blue plaques; (B) lanes 3 and 4, DNAs from the cells infected with viruses from two white plaques. Recombinant virus plaques obtained using the DNA ligation method (C) or the new homologous recombination method (D). CV-1 cells grown on six-well plates were infected with 10-fold dilutions (10^{-3} , 10^{-4} , and 10^{-5}) of viruses obtained on days 5–8 after the transfection. After 48 h, the cells were overlaid with agar medium containing the X-Gal to visualize the recombinant blue plaques.

plasmid pNZ2. Almost 100% (99.3%) of the virus plaques produced by the DNA ligation method were recombinant, and 92.2% of the recombinant virus plaques were obtained by the co-transfection-recombination method. Only 0.18% of the virus plaques were recombinant using the traditional method. An earlier study demonstrated that *lacZ* gene-based screening VV recombinants represented about 0.1% of the total viral progeny (3). Thus, both new strategies for the construction of recombinant VV were highly efficient in the generation of recombinants. This is due to the use of VV arms for transfection, which can be rescued either by ligation with the foreign DNA or by the new homologous recombination method using the transfer plasmid. Previously, when an insertion was directed into the thymidine kinase (TK) locus of the VV genome, usually between 10% and 80% of TK plaques were recombinant (3,8).

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The main advantage of these alternative methods is that any poxvirus strain, including VV itself, can be inactivated by PUV and used as a helper virus for the production of recombinant viruses. The properties of non-replicating PUV-VV have recently been reported (7,14,18; unpublished data). Once inactivated, a virus can provide the early functions required for replication and maturation of transfected viral DNA but cannot replicate and mature into infectious particles. The high efficacy of recombinant virus production and the purity of the plaques were achieved by complete digestion of VV genomic DNA at a unique *NotI* site and by the use of an inactivated helper virus. Therefore, it does not require reporter genes and multiple plaque purification cycles, which are needed for most methods of recombinant VV constructions. Furthermore, for the first time, we demonstrated that intracellular homologous recombination between DNA arms obtained at a unique restriction site and intact naked plasmids in the presence of a helper virus could rescue the genomic viral DNA to produce recombinant VV. We have recently used this strategy to generate herpesviruses as well (2). The DNA ligation method could be more convenient for the generation of recombinant viruses that express toxic gene products or when foreign DNA is inserted into a newly created unique restriction site of the VV genome. When a series of recombinant viruses carrying foreign DNA at the same locus are required, the homologous recombination method could be a better choice. An important requirement of these methods is the presence of a unique restriction site on the VV genome. Here, we took advantage of the unique *NotI* site present in most VV strains. However, other unique restriction sites can be created in the VV genome by genetic manipulation (9) and used for insertion of a foreign gene expression cassette.

In summary, we report two new methods for the fast and efficient construction of recombinant VV. Neither virus purification cycles nor reporter gene selection is necessary because 90%–100% of the VV progeny carry the foreign DNA. An important feature of these strategies is that any VV strain

can be used as a helper virus after PUV inactivation. These methods should help to advance the development of VV vector systems for vaccine and gene therapy studies. They can be also used in functional studies to investigate the virus replication cycle and packaging if various early mutants of VV are used as the helpers.

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