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Tn7-mediated Introduction of DNA into Bacmid-cloned Pseudorabies Virus Genome for Rapid Construction of Recombinant Viruses*

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Abstract: *lacZα-mini-attTn7* was inserted into the intergenic region between the gG and gD genes in a PRV bacterial artificial chromosome (BAC) by homologous recombination in *E. coli*. The resulting recombinant BAC (pBeckerZF1) was confirmed by PCR and sequencing. Green fluorescent protein (GFP) gene was then transposed into pBeckerZF1 by transposon Tn7 to generate pBeckerZF2. Recombinant viruses vBeckerZF1 and vBeckerZF2 were generated by transfection with the corresponding BAC pBeckerZF1 or pBeckerZF2. The titers and cytopathic effect (CPE) observed for by vBeckerZF1 and vBeckerZF2 was comparable to that of the parental virus vBecker3. vBeckerZF2 was serial passaged for five rounds in cell culture, and the mini-Tn7 insertion was stably maintained in viral genome. These results show that recombinant viruses can be rapidly and reliably created by Tn7-mediated transposition. This technology should accelerate greatly the pace at which recombinant PRV can be generated and, thus, facilitate the use of recombinant viruses for detailed mutagenic studies.

Key words: Pseudorabies virus (PRV); Virus construction; Tn7-mediated transposition

Pseudorabies virus (PRV), an important pathogen causing Aujeszky's disease in swine and leading to latent infection (6), is a member of the *Alphaherpesvirinae* subfamily and could served as a useful model organism for the study of herpesvirus biology (9). Like most of the herpesviruses, PRV has a very large double stranded DNA genome (~143kb) with more than 70 genes identified (3). Functional characterization of these viral genes, by generating null mutants and investigating the resulting changes in phenotype, is very important in understanding the molecular aspects of herpesvirus replication and pathogenesis. For some key genes, smaller targeted mutations are required to identify the distinct

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functional domains within the proteins (9).

Such studies often require establishment of large numbers of recombinant viruses which are usually created by homologous recombination in infected cells relying on the cellular recombination and repair machinery. However, this can be a laborious and sometimes impossible task, especially if the mutant has a severe growth disadvantage compared to the wild-type virus. Bacterial artificial chromosomes (BACs), single copy F-factor-based plasmid vectors of intermediate insert capacity (15), have now enabled the cloning of complete herpesvirus genomes and infectious virus genomes can be shuttled between *Escherichia coli* (*E. coli*) and eukaryotic cells. While herpesvirus BAC DNA engineering in *E. coli* requires neither restriction sites nor cloning steps and allows the introduction of a wide variety of DNA modif-cations, the large size of these bacmids precludes the use of rapid in vitro methods of manipulation commonly employed for construction of small plasmids.

Tn7, a site-specific transposon, transposes almost exclusively to a distinct attachment site named attTn7 within the *E. coli* genome (1). By introducing this attTn7 sequence into a BAC, Tn7 can serve as an insertion vehicle (4). This is particularly useful if numerous genes and constructs need to be tested for their expression in the context of viral genome. Tn7-mediated transposition has been well exploited for research on functional genomics of baculoviruses (4). Recently, this technology has been applied to bacmid-cloned cytomegalovirus (CMV), a member of the *Gammaherpesvirinae* subfamily, for rapid recombinant virus construction (2).

In this paper, we report the development of a technology employing Tn7-mediated transposition as a rapid and reliable method for recombinant PRV construction. A *lacZα-mini-attTn7* region was inserted into the intergenic region between the gG and gD genes in an attempt to maintain every gene and element of the parental virus. Then green fluorescent protein (GFP) gene was introduced to test the utility of this transposition system and the stability of mini-Tn7 insertions in cell culture. The technology should greatly facilitate the detailed mutagenic studies of PRV.

MATERIALS AND METHODS

Plasmids, strains, and reagents

Plasmids pGS284 and pBecker3, strains GS500 and S17λπ were provided by Lynn W. Enquist, Princeton University, USA. The pGEM-T Easy was purchased from Promega Co. (Maddison, USA), and plasmid pEGFP-N1 from Clonetech Laboratories, Inc. (Mountain View, USA). DNA restriction enzymes, T4 DNA ligase, alkaline phosphatase, *exTaq* hot start DNA polymerase and 2×GC buffer I were the products of TaKaRa Biotechnology Co., Ltd. (Dalian, China). Plasmids pFBCMV-GFP and pZFBΔtk were constructed and stored in our lab. Strains DH5α and DH10B were stored in our lab. Lipofectin reagent and Delbecco's Modified Essential Medium (DMEM) were purchased from Invitrogen Co. (Carlsbad, USA), and fetal bovine serum (FBS) from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China).

Virus and cells

The wild-type PRV used was vBecker3, generated by transfection of pBecker3 into Vero cells (18). Mutant PRVs (vBeckerZF1 and vBeckerZF2) were

constructed by manipulation of pBecker3 and transfection of mammalian cells (see below). All PRV strains were propagated in the PK15 (porcine kidney 15) cell line. Virus titers were determined in duplicate by infectivity end-point assay on PK15 cells in 96-well plates following infection with 100μL/well of 10-fold serially diluted stocks. The mammalian cells were grown in DMEM supplemented with 10% FBS. Viral infections were performed in DMEM supplemented with 2% FBS. Unless otherwise indicated, all media used contained 100U /mL penicillin and 100 μg /mL streptomycin.

Construction of plasmids and BACs

Constructions of plasmids and BACs are illustrated in Fig.1. Unless otherwise noted, all PCRs were performed with *exTaq* hot start polymerase and 2×GC buffer I. The 433 bp of the upstream homology region for allele exchange in *E. coli* was amplified by PCR from pBecker3 with the primers HRattTn7U-F (5'-GAGCTCCTGCTCCTGGGCTTCCTG-3', with a *Sac*I site underlined) and HRattTn7U-R (5'-GAATTC CCCGTAAGCAAGGCCGTA-3', with an *Eco*RI site underlined) and cloned into the *Sac*I and *Eco*RI sites of pEGFP-N1 to generate pZF1, while the 448 bp of the downstream homology region was amplified with the primers HRattTn7D-F (5'-GTCGACGTGCGATC CACGCCCAGC-3', with *Sal* I site underlined) and HRattTn7D-R (5'-GGATCCATGCATGCGCCCAAA GATCTGCCTG-3', with *Bam*H I site underlined and *Nsi*I site double underlined) and cloned into the *Sal* I and *Bam*H I sites of pZF1 to generate pZF2. PCR parameters for amplification of both homology regions were as follows: 96℃ for 2 min to denature, followed by 30 cycles of 94℃ for 30 s, 70℃ for 30s, with a final 10-min extension at 72℃. The 712 bp *lacZα-mini-attTn7* region was released by *Bbs* I digestion of pZFBΔtk (unpublished) which contains an 8.6 kb *Bsu36*I fragment from pHZB10 (19), generating an *Eco*RI compatible end and a *Sal*I site. Plasmids pZF3 was produced by inserting the *lacZα-mini-attTn7* region into the *Eco*RI and *Sal*I sites of pZF2. To construct the transfer vector for allele exchange in *E. coli,* a shuttle plasmid pGS284 (17) was used, which includes the π-protein-dependent R6K origin of replication (7), the RP4oriT origin for conjugative transfer (16), and the negative selection marker sacB (11). First, pZF3 was digested by *Sac*I and dephosphorized, and pGS284 was digested by *Sac*I. Then the two linearized plasmids were ligated and the product was transformed into DH5α to select for ampicillin (Amp) resistance. Since pGS284 was unable to grow in a strain that lacks π, only the Amp resistant strains containing the hybrid plasmid pZF4 grew. Plasmid pZF4 was digested by *Nsi*I to excise the origin of replication of pEGFP-N1 and self-ligated to generate the transfer vector pZF5. Shuttle plasmid pFBCMV-GFP, a derivative of pFastBac Dual (Invitrogen), was generated by replacing the P_{10} and P_H promoters with a P_{CMV} promoter and a GFP gene cloned under the promoter.

The pBeckerZF1 BAC was generated by the sugar suicide allele exchange system in *E. coli* as described previously (17). Briefly, conjugal transfer of the allele exchange vector occurred following cross-streaking of GS500 containing pBecker3 and S17λpir (**7**) containing pZF5 on a Luria broth (LB) plate without antibiotics and incubated overnight at 30℃. Each intersection from the crossed streaks was inoculated into 5 mL of LB plus 25 μg/mL Amp and 25 μg/mL chloramphenicol (Cm) and incubated overnight at

37℃ with rotating to select for recombinants. To select for deletion of the allele exchange vector from pBecker3, 1 μL of overnight culture was inoculated into 5 mL of LB-Cm and incubated overnight at 37℃ with rotation. The culture was serially diluted (10^{-3}, 10^{-4}, and 10^{-5}), and 50 μL of each dilution was plated onto LB plates containing 5% sucrose, 25 μg/ml Cm, 40 μg/ml isopropyl-β-D-thiogalactopyranoside (IPTG) and 200 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). A single blue colony was transferred into 5 mL of LB-Cm and incubated overnight at 37℃. Miniprep DNA from 1 mL overnight culture was diluted to low concentration (~ 100ng/mL). The diluted BAC was transformed into *E. coli* strain DH10B (Invitrogen Co., Carlsbad, USA), precluding co-transformation of more than one copy BAC DNA into a single cell. After one hour incubation at 37℃, transformed cells were spread on LB plates containing Cm, IPTG and X-gal. Blue colonies were further screened for Amp sensitivity then by PCR and sequencing (described below).

The pBeckerZF2 BAC was generated by Tn7-mediated transposition as described in the instruction manual of the Bac-to-Bac™ system (Invitrogen Co., Carlsbad, USA). White colonies were picked and further screened by PCR (described below).

PCR analysis of recombinant BACs

Virus DNA was obtained from infected PK15 cells as described previously (14). The PCR reactions were set up containing 1×GC buffer I, 200 μmol/L dATP, 200 μmol/L dCTP, 200 μmol/L dGTP, 200 μmol/L dTTP, 0.5 μmol/L concentration of forward primer, 0.5 μmol/L concentration reverse primer and 1U of *exTaq* hot start polymerase with a total volume of 25μL. For analysis of pBeckerZF1 and vBeckerZF1, two primers flanking the homology regions pBeatt-Tn7-F (5'-GCCCTCTGACATCTTCGTGACCC-3') and pBeattTn7-R (5'-CGAACTTGTACGTGCGGTG CTG-3') were designed and cycling parameters were as follows: 96℃ for 2 min to denature, followed by 30 cycles of 94℃ for 30 s, 70℃ for 1min 50s, with a final 10-min extension at 72℃. For analysis of pBeckerZF2, M13 forward primer (5'-TGTAAAACG ACGGCCAGT-3') and reverse primer (5'- TCACAC AGGAAACAGCTAT GAC -3') were used with the parameters as follows: 96℃ for 2 min to denature, followed by 30 cycles of 94℃ for 30 s, 55℃ for 30 s, 72℃ for 3 min 45s, with a final 10-min extension at 72℃. The PCR products were detected by ethidium bromide staining after separation on 0.8 to 1.0% agarose gels. The PCR product, using pBeattTn7-F and pBeattTn7-R for test of pBeckerZF1, was cloned into pGEM-T Easy and further screened by sequencing.

BAC DNA preparation and reconstitution of infectious viruses

Minipreps of BAC DNA were prepared according to methods developed for the isolation of large plasmids (defined in the Instruction Manual of the Bac-to-Bac™ system/Life Technologies). BAC DNA isolated from 1mL of overnight cultures was used for transfection of 40-60% confluent PK15 cells in a 35-mm disk. Transfection was performed with Lipofectin™ reagent according to the manufacturer's instructions.

RESULTS

Construction of the pBeckerZF1 BAC

Site-specific transposition by transposon Tn7 has been developed to rapidly introduce genes or *cis* elements

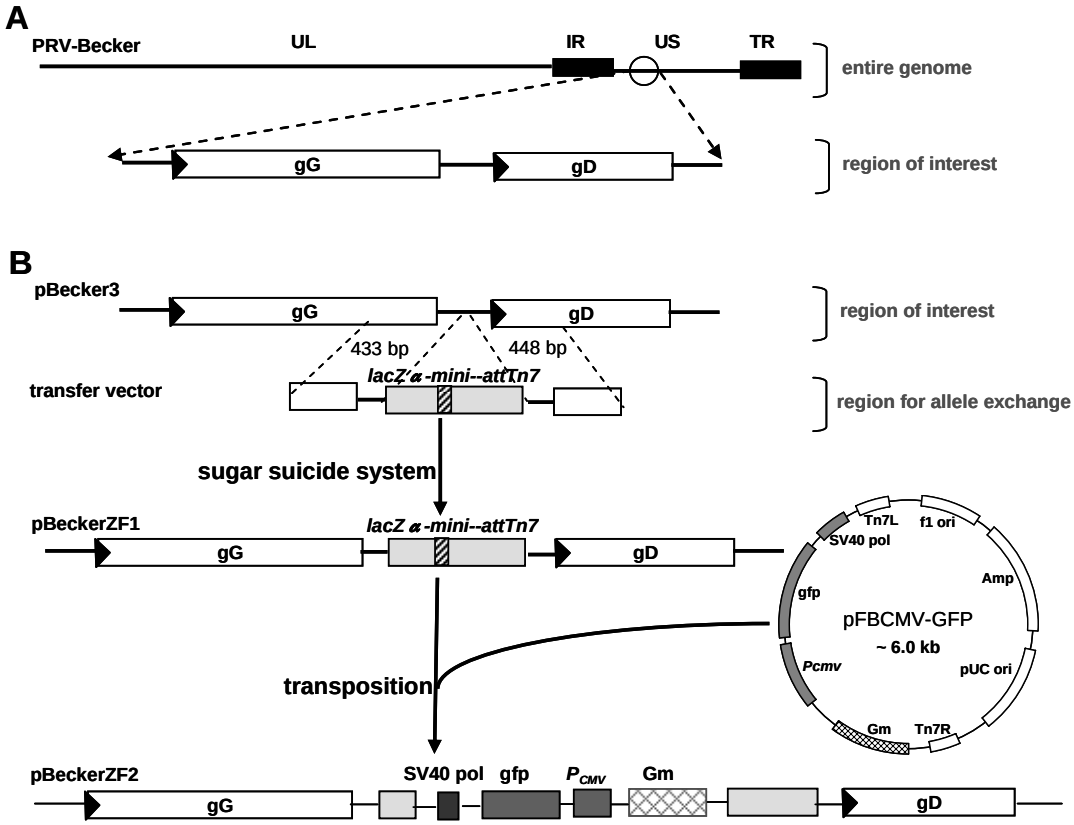

Fig. 1. Insertion of lacZα-mini-attTn7 region into pBecker3 and construction of recombinant bacmids by Tn7-mediated transposition are illustrated. A, Schematic diagram of the PRV-Becker genome depicting the unique long (UL), unique short (US), internal repeats (IR), and terminal repeats (TR) regions. A portion is expanded to show the gG and gD genes. For clarity, only this region of all subsequent viral bacmids is illustrated. B, Construction of pBeckerZF1 and pBeckerZF2. Viral genes are represented as rectangles with an arrowhead denoting the transcription direction. lacZα is represented as light gray boxes and attTn7 is represented as hatched boxes.

into viral bacmids and has proved to be a powerful tool in studies on herpesvirus genetics (2). In this study, to test whether this technology works well in PRV, a recombinant BAC pBeckerZF1 including *lacZα-mini-attTn7* was constructed using the *E. coli* recombination system, which was inserted into the intergenic sequence between the putative polyadenylation signal of the gG gene and the putative TATA signal of the gD gene (3) without loss of any viral genes (Fig.1 and Fig.3).

Insertion of *lacZα-mini-attTn7* into the target site in pBecker3 is shown in Fig.2, evidenced by the different sizes of PCR products with pBeckerZF1 or pBecker3 as template. For PCR analysis of pBeckerZF1, two

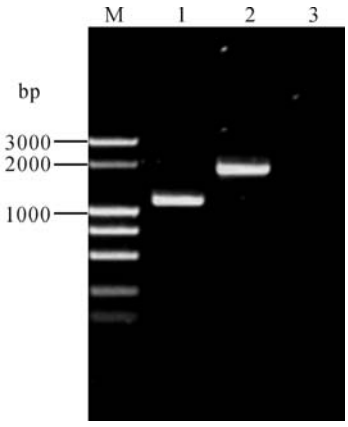

Fig. 2. PCR analysis of pBeckerZF1. Two primers flanking the homology regions were used. M, DL3000 DNA marker; 1, pBecker3 as template; 2, pBeckerZF1 as template; 3, Double distilled H_2O (ddH_2O) as template.

primers flanking the homologous regions for allele exchange in *E. coli* were designed to exclude the possibility of contamination by transfer vector pZF5. The size of PCR product with the template pBeckerZF1 is about 710 bp larger than that with pBecker3, which is corresponds exactly with the size of *lacZ-mini-attTn7* region (Fig.2). Further sequencing of the PCR product with the template pBeckerZF1 established that the *lacZα-mini-attTn7* region was inserted 44bp downstream from the putative polyadenylation signal of gG and 23bp upstream from the putative TATA signal of gD (Fig.3.).

Transfection of pBeckerZF1 and characterization of vBeckerZF1 in cell culture

To test whether viruses vBeckerZF1 generated from the pBeckerZF1 transfection included the *lacZα-mini-attTn7* region, PCR was performed with the same primers used for establishing pBeckZF1 was constructed correctly (see above). The size of PCR product with the vBeckerZF1 genome DNA as the template is about 710 bp larger than that obtained with vBecker3 (data not shown). Therefore, transfection of pBeckerZF1 resulted in the production of virus containing the lacZα-mini-attTn7 region, vBeckerZF1, without the need for further plaque purification.

To examine the growth properties of vBeckerZF1, PK 15 cells were transfected in parallell, with pBeckerZF1 or pBecker3, respectively. Viral titers of the resulted vBeckerZF1 were typically in the order of 10^8 to 10^9 $TCID_{50}$/mL, which is comparable to that

GCCCTCTGACATCTTCGTGACCCCCACCGGCAGCCCCGCCCTGCTCCTGGGCTTCCTGGGCAGCGCGCTCGCCTCGCGCCCCCTGCA

CCTGACGGCCGGGGAGACGGCCCAGCACGTGCGCGAGGCCCAGCAGAAGAGCCGCCACATCCGCTCCCTCGGCGGCCTCCAGCTC

TCGGTCGAGACCGAGACCACCAACACCACCACCCAGACGGGCCTGTCGGGCGACATCCGCACCTCGATCTACATCTGCGTCGC

CCTCGCCGGCCTGGTCGTCGTGGGCATCGTCATCATGTGCCTCCATATGGCGATCATCAGGGCCCGGGCCCGGAACGACGGCTACCG

polyadenylation signal

CCACGTGGCCTCCGCCTGACCCGGCCCCGCCCGACTCCCCCGCGATTCCCCCCCTCTCTCACCGGGTGTCCATCTTCAATAAAGTAT

GTCTCAAACACCTAATTTGCGTACGGCCTTGCTTACGGGG**AATTAAGTCTTCGAACCAATACGCAAACCGCCTCTCCCCGCGCG**

TTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAG

TTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAAT

TTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCC

ACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCACATAACAGGAAGAAAAATGCCCCGCTT

attTn7

ACGCAGGGCATCCATTTATTACTCAACCGTAACCGATTTTGCCAGGTTACGCGGCTGTCGACCTCGAGGGGGGGCCCGGTA

CCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCG

TTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTC

CCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGC

TATA signal

GCTTCGAAGACGCTCGACGTGCGATCCACGCCCAGCGGTCCATAAAATTGGGTTGGCGCCCCAGGTTCCCATACACTCACCCGCC

AGCGCCATGCTGCTCGCAGCGCTATTGGCGGCGCTGGTCGCCCGGACGACGCTCGGTGCGGACGTGGACGCCGTGCCCGCGCCGAC

CTTCCCCCCGCCCGCGTACCCGTACACCGAGTCGTGGCAGCTGACGCTGACGACGGTCCCCTCGCCCTTCGTCGGCCCCGCGGACG

TCTACCACACGCGCCCGCTGGAGGACCCGTGCGGGGTGGTGGCGCTGATCTCCGACCCGCAGGTGGACCGGCTGCTGAACGAGGC

GGTGGCCCACCGGCGGCCCACGTACCGCGCCCACGTGGCCTGGTACCGCATCGCGGACGGGTGCGCGCACCTGCTGTACTTTATCG

AGTACGCCGACTGCGACCCCAGGCAGATCTTTGGGCGCTGCCGGCGCCGCACCACGCCGATGTGGTGGACCCCGTCCGCGGACTAC

ATGTTCCCCACGGAGGACGAGCTGGGGCTGCTCATGGTGGCTCCGGGGCGGTTCAACGAGGGCCAGTACCGGCGCCTGGTGTCCGT

CGACGGCGTGAACATCCTCACCGACTTCATGGTGGCGCTCCCCGAGGGGCAAGAGTGCCCGTTCGCCCGCGTGGACCAGCACCGC

Fig. 3. Sequence analysis of lacZ-mini-attTn7 insertion into pBekcer3. *lacZ-mini-attTn7* region is in bold and the attTn7 is framed. Homology regions for allele exchange are underlined with polyadenylation signal for gG and TATA signal for gD double underlined.

obtained for vBecker3. The CPE observed for vBeckerZF1 was also indistinguishable from that observed for vBecker3. Together, these data indicated that the insertion of *lacZα-mini-attTn7* in the intergenic region between the gG and gD genes did not visibly affect the viral growth at recorded levels of viral titers and CPE.

Tn7-mediated transposition for introduction of sequences into PRV bacmid

Three components are necessary for Tn7-mediated transposition: an attachment site, attTn7, a shuttle plasmid (pFastBac Dual or its derivatives) including both the right and left arms of Tn7, and a third plasmid (pMON7124) encoding the necessary *trans*-acting factors required in transposition (4). In this study the *lacZα-mini-attTn7* region containing an attTn7 site in lacZα gene without disrupting its function was used (4). To evaluate the utility of Tn7-mediated transposition for rapid construction of the recombinant virus, a derivative shuttle vector pFastBacCMV-G containing the GFP gene under P_{CMV} was transformed into DH10B harboring both pBeckerZF1 and pMON7124. Transformants were selected on a plate containing Cm, X-Gal and IPTG. White colonies were further screened by PCR with the two primers flanking the attTn7 site. Integration of mini-Tn7 is shown in Fig.4. The PCR product with pBeckerZF2 as a template was 3.5 kb (lane 2) in length as expected, while that with pBeckerZF1 carrying the intact attTn7 as template was about 300 bp in length (lane 3). Transfection of pBeckerZF2 resulted in production of infectious recombinant virus vBckerZF2 with GFP expression (Fig.5).

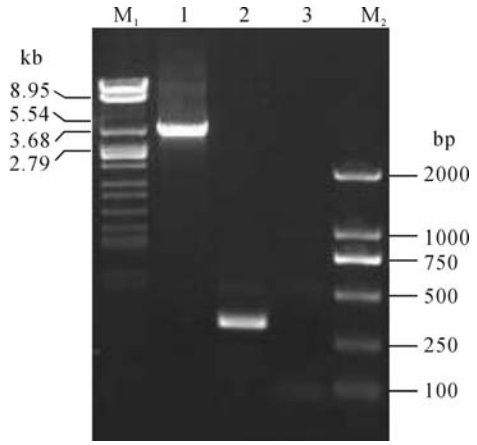

Fig. 4. PCR analysis of pBeckerZF2. M13 primers flanking attTn7 were used. M_1, λ DNA/*Eco*R I+*Bam*H I+*Hin*d III; 1, pBeckerZF2 as template; 2, pBeckerZF1 as template. 3, ddH_2O as template; M_2, DL2000 DNA marker.

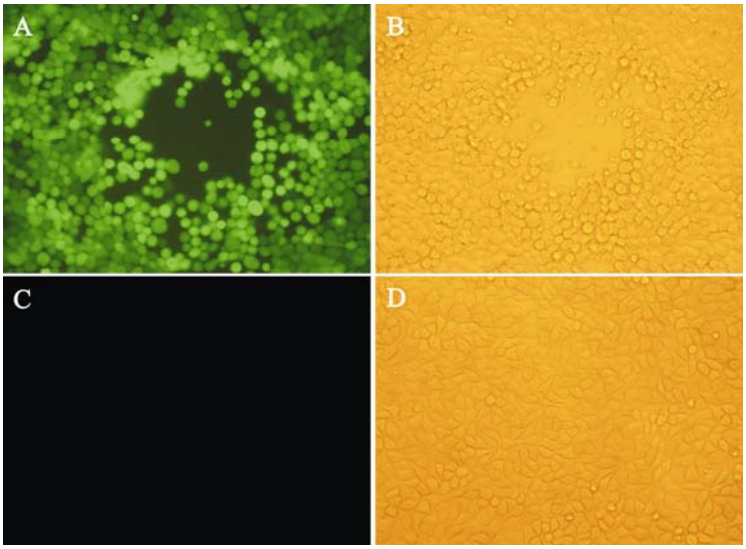

Fig. 5. Recovery of infectious vBeckerZF2 by transfection. Pictures were taken 48 hours post transfection. Recovery of vBeckerZF2 was monitored by CPE and GFP expression. **A**: PK15 cells transfected with pBeckerZF2 exposed to ultraviolet light; **B**: PK15 cells transfected with pBeckerZF2 exposed to visible light; **C**: Normal PK15 cells exposed to ultraviolet light; **D**: Normal PK15 Cells exposed to visible light.

To test whether the mini-Tn7 insertion impaired viral growth, a strategy for examining the growth above). Viral titers and CPE developed by vBecker-ZF2 were indistinguishable from that developed by vBecker3. So insertion of at least 3.5 kb mini-Tn7 region into the attTn7 site in vBeckerZF1 did not visibly affect the viral growth at levels of viral titers and CPE.

Stability of mini-Tn7 insertions in vBeckerZF1

For Tn7-mediated transposition to be useful in herpesvirus genetics, the insertions must be stable in the recombinant virus resulting from transfection. In this study, the stability of mini-Tn7 was tested in cell culture. vBeckerZF2 harvested from transfection of pBeckZF2 was serial passaged in PK15 cells for five rounds at a low motility of infection (MOI) (~0.01 $TCID_{50}$/cell). The virus harvested was applied to a plaque assay for GFP expression. Of 200 plaques counted, 100% expressed the GFP. This indicates that a 3.5 kb mini-Tn7 insertion by Tn7-mesitad transposition in vBeckerZF2 is stable in cell culture.

DISCUSSION

Members of the *Herpesviridae* family are characterized with large (130~230kb) double stranded DNA genomes capable of encoding as many as 200 potential genes (12). The genetic analysis of these complex viruses has been a challenge to herpesvirologists. Successful techniques for gene- ration of recombinant herpsviruses have been developed by homologous recombination between the viral genomes and the plasmids containing the desired mutations in infected cells (8, 10). However, these techniques are limited by the fact that wild-type virus is invariably present during the selection process, and, therefore, extensive purification is required to isolate the recombinant virus (5). Mutations which confer a growth disadv- antage to the recombinant virus may make purifi- cation very problematic (5). BAC technology has undoubtedly opened new avenues for mutagenesis of these large virus genomes and enabled the systematic mining of the information stored in virus genomes. The resultant mutant bacmids are clonal and free of contaminating wild type sequences, facilitating rapid production of pure recombinant viruses (2). While this represents a significant advance, mutant construction of these large size bacmids is still time consuming es-pecially when detailed mutagenesis are needed for structure-function studies of viral proteins.

To address this problem, Gabriele Hahn *et al* adapted a technology employing Tn7-mediated site-specific transposition as a rapid and highly reliable method to create mutants of cytomegalovirus (2). In the present study, we engineered the *lacZα -mini-attTn7* region into PRV BAC by homologous recombination in *E. coli*, and recombi- nant viruses could then be created by transposition of a mini-Tn7 region containing the desired DNA sequences into the attTn7 site. This technology should greatly facilitate detailed mutagenesis of PRV since most of the effort of recombinant virus production will now be located at the point of plasmid construction of the desired mutations.

Since our primary interest lies in functional genomics of PRV, the emphasis of our design was to maintain every gene and element of the parental virus. Thus the *lacZ-mini-attTn7* region should be positioned in the viral genome, so as not to interfere

with expression of viral genes or function of *cis* elements. This is not necessarily a straightforward task since the herpesvirus genomes are very compact with few regions that are not transcribed (13). After consideration we therefore chose to place the *lacZα-mini-attTn7* region in a relatively large intergenic space between the gG and the gD genes (3). After transfection, the recombinant virus vBeckerZF1 (carrying the *lacZα-mini-attTn7* region) and the vBeckerZF2 virus (carrying the 3.5 kb mini-Tn7 insertion region) were recovered and their growth properties were found to be indistinguishable from that of the wild-type virus vBecker3 at the examined levels of viral titers and CPE. Nevertheless, further studies are required to determine the growth properties of vBeckerZF1 and vBeckerZF2 in tissue cultures and their virulence in animals.

Stability of the insertions in recombinant viruses generated by BAC technology should be taken into consideration. In studies reported by Smith and Enquist, F-plasmid sequences inserted in the gG ORF of PRV were unstable following passage, however, the lacZ insertion at the same locus or the F-plasmid insertion at another locus showed no signs of instability (17, 18). More information is needed to understand these differences. We tested the stability of mini-Tn7 insertions in the intergenic region between the gG and gD genes and results showed that insertion of a 3.5 kb mini-Tn7 into vBecker1 by transposition was stable in cell culture. Future work is needed to make clear whether larger insertions or insertions of different sequences into vBeckerZF1 are still stable.

The size of insertions should also be taken into consideration. It is yet unclear how many heterologous genes may be packaged into the BAC-derived viruses before size constraints become problematic. Although HSV can package up to 30 kb in excess of its genome, the packaging constraints of other herpesviruses are yet unknown. Initial studies with MCMV BACs did report stability problems once more than 5 kb of extra DNA was inserted into the viral genome (5). In this study, to minimize the size of insertion, the *lacZα-mini-attTn7* region was inserted alone but not together with an antibiotic resistance gene (which is standard for homologous recombination in *E. coli*). Although more complicated procedures were needed to get the clonal recombinant bacmid, this did permit a capacity for accommodating larger insertions.

In summary, a technology employing Tn7-mediated site-specific transposition as a rapid method to introduce novel sequences into bacmid-cloned PRV genome was developed and which allowed recombinant viruses to be rapidly and reliably created. This technology should accelerate greatly the pace at which recombinant viruses can be created and, thus, facilitate the use of recombinant viruses for detailed mutagenic studies.

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References

1. **Craig N L.** 1996. Transposon Tn7 . **Curr Top Microbiol Immunol,** 204 (1): 27-48.
2. **Hahn G, Jarosch M, Wang J B, *et al.*** 2003. Tn7-mediated introduction of DNA sequences into bacmid-cloned cytomegalovirus genomes for rapid recombinant virus construction. **J Virol Methods,** 107: 185-194.
3. **Klupp B G, Hengartner C J, Mettenleiter T C, *et al.*** 2004. Complete, annotated sequence of the pseudorabies virus genome. **J Virol,** 78 (1): 424-440.
4. **Luckow V A, Lee S C, Barry, G F, *et al.*** 1993. Efficient generation of infectious recombinant baculovirus genome propagate in *Escherichia coli*. **J Virol,** 67 (8): 4566-4579.
5. **MacGregor A, Schleiss M R.** 2001. Recent advances in herpesvirus genetics using bacterial artificial chromosomes . **Mol Genet Metab**, 72 (1): 8-14.
6. **Mettenleiter T C.** 2000. Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis-state of the art, June 1999. **Vet Res,** 31 (1): 99-115.
7. **Miller V L, Mekalanos J J.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. **J Bacteriol,** 170 (6): 2575-2583.
8. **Mocarski E S, Post L E, Roizman B.** 1980. Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. **Cell,** 22 (1): 243- 255.
9. **Pomeranz L E, Reynolds A E, Hengartner C J.** 2005. Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. **Microbiol Mol Bio Rev,** 69 (3): 462-500.
10. **Post L E, Roizman B.** 1981. A generalized technique for deletion of specific genes in large genomes: alpha gene 22 of herpes simplex virus 1 is not essential for growth . **Cell,** 25 (1): 227-232.
11. **Ried J L, Collmer A.** 1987. An nptI-sacB-sacR cartridge for constructing directed, unmarked mutations in gram-negative bacteria by marker exchange-eviction mutagenesis. **Gene,** 57 (2-3): 239-246.
12. **Roizman B.** 1993. The *herpesviridea*, a brief introduction. In: Roizman B, Whitley R J, Lopez C. (eds), The human herpesviruses . **Raven Press, New York,** p 1-9.
13. **Roizman B, Sears E.** 1996. In: Fields B N, Knipe D M, Howley PM. (eds), Fundamental virology. **Lippincott-Raven, Philadelphia,** pp. 1043-1107.
14. **Schwartz J A, Brittle E E, Reynolds A E, *et al.*** 2006. UL54-null pseudorabies virus is attenuated in mice productively infects cells in culture . **J Virol,** 80 (2): 769-784.
15. **Shizuya H, Birren B, Kim U J, *et al.*** 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. **Proc Natl Acad Sci USA,** 89 (18): 8794-8797.
16. **Simon R, Priefer U, Pühler A.** 1983. A broad host range mobilization system for in vivo genetics engineering: transposon mutagenesis in gram negative bacteria. **Biotechnology** 1, 784-791.
17. **Smith G A, Enquist L W.** 1999. Construction and transposon mutagenesis in *Escherichia coli* of a full-length infectious clone of pseudorabies virus, an *alphaherpesvirus* . **J Virol,** 73(8): 6405-6414.
18. **Smith G A, Enquist L W.** 2000. A self-recombining bacterial artificial chromosome and its application for analysis of herpesvirus pathogenesis. **Proc Natl Acad Sci USA,** 97 (9): 4873-4878.
19. **Wang H Z, Deng F, Pijlman G P, *et al.*** 2003. Cloning of biological active genomes from a Helicoverpa armigera single-nucleocapsid nucleopolyhedrovirus isolate by using a bacterial artificial chromosome. **Virus Res,** 97: 57-63.