Auto-excision of bacteria artificial chromosome facilitated by terminal repeat-mediated homologous recombination: a novel approach for generating traceless genetic mutants of herpesviruses

Running title: Auto-excisable RRV BAC

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Key words: Herpesvirus, Bacteria artificial chromosome, Auto-excision, Terminal repeat-mediated homologous recombination, RRV
Abstract

Infectious bacterial artificial chromosomes (BAC) of herpesviruses are powerful tools for genetic manipulation. However, the presence of BAC vector sequence in the viral genomes often causes genetic and phenotypic alterations. While the excision of the BAC vector cassette can be achieved by homologous recombination between extra duplicate viral sequences or loxP sites-mediated recombination, these methods are either inefficient or leave a loxP site mark in the viral genome. Here, we describe the use of viral intrinsic repeat sequences, which are commonly present in herpesviral genomes, to excise the BAC vector cassette. Using a newly developed in vitro transposon-based cloning approach, we obtained an infectious BAC of rhesus rhadinovirus (RRV) strain RRV26-95 with the BAC vector cassette inserted in the terminal repeat (TR) region. We showed that the BAC vector cassette was rapidly excised upon reconstitution in cells predominantly through TR-mediated homologous recombination. Genetic and phenotypic analysis showed that the BAC-excised virus was reversed to wild-type RRV. Using this auto-excisable BAC clone, we successfully generated a RRV mutant with a deletion of Orf50, which encodes a replication and transcription activator (RTA) protein. Together, these results illustrate the usefulness of TR for genetic manipulation of herpesviruses when combined with the novel transposon-based cloning approach.
Introduction

Molecular cloning of large DNA viruses, particularly herpesviruses, as bacterial artificial chromosomes (BAC) has greatly facilitated their genetic manipulation. Since the cloning of mouse cytomegalovirus (MCMV) genome as the first herpesviral infectious BAC (20), over a dozen of DNA viruses have been cloned as BACs in the last decade including human cytomegalovirus, herpes simplex virus type 1, Epstein-Barr virus (EBV), Kaposi’s sarcoma-associated herpesviruses (KSHV), pseudorabies virus, Marek’s disease virus, murine gammaherpesvirus-68 (MHV-68), rhesus rhadinovirus (RRV), varicella-zoster virus (VZV), guinea pig cytomegalovirus, and vaccinia virus as some examples (2, 6, 9, 10, 13, 14, 19, 25, 28-30, 35, 38). These recombinant BACs have become important tools for studying the functions of viral genes (1, 21). However, the insertion of a BAC vector cassette into the viral genome often leads to genetic and phenotypic alterations of the virus because of the strict size requirement of the viral genome for packaging and stability, and the disruption of the viral genome resulting from the insertion event (3, 5, 32). Several systems have been developed to excise the BAC vector cassette. One of them is to insert a fragment of identical viral sequences on the opposite side of the BAC cassette, which allows the recombination between two duplicate flanking sequences and excision of the BAC vector cassette in mammalian cells (32). While this approach has been shown to be successful for MCMV, it is time-consuming requiring multiple passages and plaque purification in mammalian cells (32). The Cre-loxP system has also been successfully adapted to excise the BAC cassette (27). However, this approach, while efficiently removes the BAC cassette, still leaves a loxP site in the viral genome. Studies have shown that this 34-bp sequence is sufficient
to cause phenotypic alterations to the virus (36). A recent study has shown that an
inverted duplication of viral genomic sequences within the mini-F replicon can lead to
markerless excision of the vector sequence upon reconstitution in mammalian cells (30).
Although the excision is efficient, the introduction of the inverted viral sequences makes
it complicated to generate viral mutants that have the mutated sequences adjacent to
the BAC vector sequences. Thus, an alternative approach is needed to facilitate the
excision of the BAC vector cassette and improve the BAC system for herpesviral
genetic manipulation. Since almost all the herpesviruses have either internal repeat or
terminal repeat (TR) sequences, in this study, we explore the use of these sequences
for excising the BAC vector cassette from the viral genome.

RRV is closely related to KSHV (18), a gammaherpesvirus associated with AIDS-
related malignancies including Kaposi’s sarcoma (KS), primary effusion lymphoma
(PEL), and multicentric Castleman’s disease (MCD) (16). Two RRV strains have been
independently isolated by two laboratories, RRV26-95 and RRV17577, both of which
have similar genetic composition and laboratory phenotype (11, 26). While a BAC-based
genetic system has been developed for KSHV (38), the lack of a robust replication
system and an infection animal model has rendered the genetic and pathogenic
characterizations of KSHV challenging. In contrast to KSHV, RRV replicates robustly in
rhesus fibroblasts, and induces B cell hyperplasia and MCD-like disease in SIV-
coinfected rhesus monkeys (11, 23, 33). As a result, RRV has been used as an animal
model for studying KSHV infection and pathogenesis (22). Two systems have been
developed for RRV genetic manipulation. One is based on the transfection of
overlapping cosmids into permissive cells to generate viral mutants for RRV26-95 (4)
while the other one is based on the cloning of RRV17577 genome as a BAC (14). In this report, we describe the use of a recently developed transposon-based insertion approach (37) to clone the RRV26-95 genome as a BAC. We successfully obtained a RRV BAC clone with the insertion of the BAC vector cassette in the TR region. Our results showed that the BAC vector cassette was rapidly lost from the viral genome upon reconstitution in cells. We successfully used this BAC clone to generate a RRV mutant with a knock out of Orf50 encoding a replication and transcription activator (RTA) protein. These results showed that the TR region can be used for genetic manipulation of herpesviruses when combined with the transposon-based cloning approach.

Materials and Methods

Cell and virus

RRV strain RRV26-95, kindly provided by Dr. Ronald C. Desrosiers at New England Primate Research Center, was grown in primary rhesus fibroblasts in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 50 µg/ml of gentamycin as previously described (11).

Cloning of RRV as an infectious BAC

We used a recently developed transposon-based approach to clone the RRV genome as infectious BAC (37). Briefly, an in vitro transposition reaction was performed according to the manufacturer’s instructions (Epicentre, Madison, USA). The reaction contains RRV genome, Tn5 recognition mosaic sequence-flanking BAC vector pHAIME and EZ-Tn5 transposase. The reaction product was electroporated into rhesus
fibroblasts at 500\mu F, 230\,V using a Gene Pulser II electroporation system (Bio-Rad Laboratories, Richmond, CA). Virus preparations collected from cultures that showed complete cytopathic effect (CPE) by three cycles of freezing and thawing were used to infect rhesus fibroblasts. At 18 h post infection (hpi), circular viral DNA was isolated to transform *E. coli* DH10B by electroporation at 1.8 kV, 100 \mu \Omega, and 25 \mu F in a 0.1-cm cuvette. The bacteria were plated on Luria-Bertani (LB) agar plates with 12.5 \mu g/ml of chloramphenicol. BAC DNA preparations isolated from grown colonies were analyzed by restriction digestion. DNA preparations from selected BAC clones were electroporated into rhesus fibroblasts to determine whether they were infectious based on CPE development. The insertion location of the BAC vector cassette pHAIME was determined by DNA sequencing using primers PLHAF and PHAR as previously described (37). One of the clones BAC17 with the BAC vector cassette inserted in the RRV TR region was used for this study.

**Generation of BAC17 derivatives**

To retrofit BAC17 with a GFP cassette, a plasmid pKG was constructed by inserting a GFP cassette and a Kanamycin-resistance (Kan) cassette into pMOD™-3<R6K\gamma/ori/MCS> (Epicentre, Madison, USA). A CMV-driven GFP cassette together with a Kan cassette were amplified by PCR from pKG with primers KGF: 5'-CGG TAT TTT TTG AGT TAT CGA GAT TTT CAG GAG CTA AGG AAG CTA AAA TGA GCC ATA TTC AAC GGG AAA C-3' and KFR: 5'-GCC CAG CGT CTT GTC ATT GGC GAA CTC GAA CAC GCA GAT GCA GTC GGG GCG AGT AAT CAA TTA CGG GGT CAT TAG TTC-3', where the underlined sequences were from the BAC vector cassette.
intended for homologous recombination. Recombination of the RRV BAC with the PCR product led to the replacement of guanine phosphoribosyltransferase (GPT) expression cassette and chloramphenicol resistance gene by the GFP and Kan cassettes (37). The resulting BAC clone was designated as BAC17GK.

To investigate the possible mechanism mediating BAC cassette loss, we used similar strategy to generate an infectious RRV BAC containing GPT, GFP and Kan cassettes by replacing the chloramphenicol resistance cassette with GFP and Kan cassettes. Again, a CMV-driven GFP cassette together with a Kan cassette were amplified by PCR from pKG with primers AGFP: 5’-

TTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAAGTAATCA
ATTACGGGGTCATTAGTTC-3’ and KGF: 5’-
CGGGCGTATTTTTTGAGTTATCGAGATTTTCAGGAGCTAAGGAAGCTAAAAATGAGC
CATATTCAACGGGAAAC-3’, where the underlined sequences were from the BAC vector cassette intended for homologous recombination. Recombination of the RRV BAC with the PCR product led to the replacement of chloramphenicol resistance gene by the GFP and Kan cassettes. The resulting BAC clone was designated as BAC17GGK.

Isolation of excised BAC cassette DNA molecules from BAC17GGK-infected cells

Virions of BAC17GGK were produced by transfection of rhesus fibroblasts with BAC17GGK DNA isolated from bacteria. Virions were used to infected rhesus fibroblasts in the presence of 100 µg/ml mycophenolic acid (MPA) and 25 µg/ml xanthine in order to enrich viruses containing the BAC cassette. To isolate the excised DNA molecules...
BAC cassette DNA molecules from the BAC17GGK-infected rhesus fibroblasts, total 1 DNA was treated with DNA polymerase in the presence of 1 mM dNTPs and transformed into *E. coli* DH10B by the heat shock method with or without ligation with T4 DNA ligase. DNA preparations isolated from colonies were subjected to dual restriction digestion with EcoRI and BamHI, or single digestion with BgIII.

**Southern-blot hybridization**

BAC DNA digested with indicated restriction enzymes were separated on 0.8% agarose gels, transferred to Zeta-Probe GT membranes (Bio-Rad), and hybridized with specific probes, which were labeled with $^{32}$P using the Rediprime™ II DNA Labeling System (GE Healthcare, Piscataway, NJ) and purified with mini Quick Spin DNA Columns (Roche, Nutley, NJ). Prehybridization and hybridization were done at 65°C in Rapid-Hyb buffer (GE Healthcare). The membranes were washed with 2XSSC containing 0.1% SDS for 10 min at 65°C once and 0.1XSSC containing 0.1% SDS at 65°C twice, each for 20 min. Specific signals were captured with a Typhoon scanner (GE Healthcare).

**Indirect immunofluorescence assay (IFA)**

Mock or RRV-infected cells grown on coverslips were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10 min, permeabized with 0.5% Triton X-100 in PBS (pH7.4) for 20 min, and blocked with 10% normal goat serum in DMEM for 30 min, all at room temperature. The coverslips were incubated with primary antibodies diluted with 10% normal goat serum in DMEM for 1 h at 37°C. A rabbit polyclonal antibody
(1:100) was used to detect the GFP protein, and a mouse monoclonal antibody (1:100) was used to detect the RRV glycoprotein B (gB) protein. The coverslips were incubated with a secondary goat anti-rabbit IgG Alexa Fluor 488 conjugate (1:150) or goat anti-mouse IgG2b Alexa Fluor 568 conjugate (1:150) for 45 min at 37°C. The cells were stained for nuclei by incubating with DAPI for 5 min.

Quantitative real-time PCR (qPCR)

qPCR was performed as previously described (34). Viral DNA was isolated from 200 µl of cell-free culture supernatant using the QiaAmp DNA Blood Mini Kit according to the manufacturer's protocol (QIAGEN, Valencia, CA). Ten-fold serial dilutions of pROrf50 and pKan ranging from 2 x 10^2 to 2 x 10^9 copies/ml were used to generate a standard curve for analyzing the copy numbers of RRV Orf50 and Kan, respectively.

Plasmid pROrf50 was constructed by inserting RRV RTA (RRTA) into a cloning vector SK(+) while pKan is a plasmid with a Kan cassette. qPCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a 7500 Real-time PCR System (Applied Biosystems). The Kan cassette was detected with primers qKanF: 5’- TCGGAATCGCAGACCGATA -3’ and qKanR: 5’- ACTCACCGAGGCAGTTCCAT-3’. The Orf50 gene was detected using primers qRRTAF: 5’-AAAACGACGACGACATGCTA-3’ and qRRTAR: 5’- TCCTCATTGTCGAGTTGCT-3’. The reaction consists of 7.5 µl Power SYBR® Green PCR Master Mix, 2.5 µl of each primer at 2.25 µM, 5 µl of DNA template. Amplification was performed using the following conditions: 50°C for 2 min, then 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 60 s. Reactions were performed in
triplicate and reactions without template were included as controls in the analysis. RRV genomic copy numbers were calculated based on the standard curve using the 7500 Real-time PCR System software (Applied Biosystems).

**Virus titration**

Culture supernatants containing infectious virions were subjected to 10-fold serial dilution in DMEM containing 20% FBS. Rhesus fibroblasts plated at 2x10⁴ cells per well of 96-well plates were inoculated with 50 µl/well of the diluted virus or culture medium alone as negative control. Ten replicate wells were used for each dilution. After incubation at 37°C for 2 h, 150 µl/well of DMEM was added, and CPE was examined daily. TCID⁵₀ was determined by using the method of Reed and Muench.

**Establishment of a RRTA expressing cell line**

Rhesus fibroblasts were seeded onto six-well plates and infected with pBABEhTERT retrovirus (a kind gift from Dr. Woody Wright at the University of Texas Southwestern Medical Center, Dallas) and selected in DMEM supplemented with 10% FBS and 5 µg/ml puromycin to obtain puromycin-resistant cell lines Telo-RFs. Plasmid p3XFlag-Bsd used for the expression of 3XFlag fusion proteins was constructed by replacing the neomycin-resistant gene cassette of plasmid p3XFlag (8) with a blasticidin resistant gene cassette. RRTA was amplified by RT-PCR from total RNA isolated from RRV-infected Rhesus fibroblasts using primers RRTAF1: 5'-TTT TCT AGA GGA TAC CGA CGA CAA TCA GGG TG-3' and RRTAR: 5'-TTT GGA TCC TCA AGG CGA TCC GTG GCC GCT AGA TGG-3', where the underlined sequences are NheI and BamHI.
restriction sites. The amplified product was digested with NheI and BamHI and cloned into p3XFlag-Bsd to produce RRTA expression plasmid p3Flag-RRTA-Bsd. This construct has a 3XFlag fused in-frame at the N-terminal of RRTA. RRV RTA-expressing cell lines were established by transfection of Telo-RFs with p3Flag-RRTA-Bsd, and selected with 5 µg/ml of blasticidin and 5 µg/ml of puromycin.

Generation of a RRV mutant with Orf50 deleted

BAC17GK was used to generate a RRV mutant with Orf50 replaced with a mRFP1 cassette by a two-step recombination approach (31). The zeocin resistance (Zeo) cassette was amplified from pEM7/Zeo (Invitrogen, Carlsbad, CA) with primers FZeoF: 5′-GGAGGATCCGATTACAAGGATGACGACGATAAGTAGGATAACAGGGTAATTGTT-3′ and FZeoR: 5′-TTCGATATCTAGGGATAACAGGGTAATCAGTCCTGCTCCTCGGCCACGAAGTGCACTGC-3′, where the I-SceI sites are underlined and a Flag encoding sequence is italicized. The PCR product was cut with BamHI and EcoRV, and cloned into the BglII and NruI sites of the pcDNA3 vector (Invitrogen) to generate plasmid pEPZeo-S. Plasmid pRSETB-mRFP1-Zin was generated by inserting a Zeo-I-SceI cassette into the PstI site of pRSETB-mRFP1 (7). The mRFP1 Orf has a single PstI site. The Zeo-I-SceI cassette was PCR-amplified from pEPZeo-S with primers PstI-ZF: 5′-TCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTTAGGGATAACAGGGTAATTGTTGAA-3′ and ZR-PstI: 5′-TTTCTGCAGTCAGTCCTGCTCCTCGGCCACGAAGTGCACTGCC-3′, where the underlined
sequences is a PstI site. The first recombination fragment was PCR-amplified from pRSETB-mRFP-Zin using primers RRTA-mRFP1F1: 5’-GATTTAAAAGATGGTCTTTCCCGTAATGGCGACTCATGGTCGTGTTTTAACGTTCACAGGGTGCCCTCCTCCGAGGACGTCATCAAG-3’ and RRTA-mRFP1R: 5’-CCGCCGGGTTGCGTGGGACTGTCTCGGTGGTGTGATCTTGCGTCTTGGGGTCACTC TTTCAGGCCGCCGGTGGAGTGGCGGCCCTCGGC-3’. The second recombination was performed as previously described (31).

To examine whether the recombination events had occurred as expected, PCR amplification was performed using a set of primers outside the RRTA coding frame SRRAF1: 5’-GCGACTCATGGTCGTGTTTTAACGTTC-3’ and SRRAR1: 5’-TGTGATCTTGCGTCTTGGGGT-3’, which amplified a product of 1,771 bp from the BAC17GK genome and a product of 736 bp from the RTA mutant genome, respectively.

Results

Generation of an infectious RRV BAC with the BAC vector cassette inserted in the TR

To clone the RRV genome as BAC, we carried out in vitro transposition cloning as described in the Materials and Methods, and isolated several RRV BAC clones. Upon reconstitution in rhesus fibroblasts, these BAC cones caused CPE (data not shown), indicating that they were infectious. Direct DNA sequencing identified one clone designated BAC17 with the BAC vector cassette inserted in the RRV TR region. Restriction digestion of BAC17 DNA with HindIII and EcoRI showed that its patterns were similar to that of wild type (WT) RRV26-95 (Fig. 1A). Southern-blot hybridization
with the BAC vector cassette pHAI probe detected one HindIII band and two EcoRI bands in the BAC17 lanes but not in the WT RRV lane (Fig. 1B). Since these bands contained the TR sequences, we were not able to precisely estimate their sizes. In contrast, the RRTA probe detected a 2.96 Kb HindIII band and a 7.25 Kb EcoRI band in both the BAC17 and WT RRV lanes as expected (Fig. 1C). These results indicated that the infectious BAC17 was derived from WT RRV.

**RRV BAC clone with the BAC vector cassette inserted in TR is stable in bacteria**

The TR region has multiple copies of TR unit and high G+C content, which could render the BAC cassette unstable in bacteria, and thus undesirable for genetic manipulation. We examined the stability of BAC17 by serial passage in *E. coli* strain DH10B. Specifically, we inoculated 1 \( \mu l \) of BAC17 bacteria culture into 3 ml of new LB medium containing 12.5 \( \mu g/ml \) of chloramphenicol daily for 13 passages. BAC DNA were isolated from each passage and analyzed by restriction digestion. As shown in Fig. 2A, BAC17 from all the passages had the same restriction pattern. Southern hybridization with either pHAI or RRTA probe detected the same bands in the lanes of BAC DNA from all passages (Fig. 2B and C). These results indicated that BAC17 was stable in bacteria.

**Rapid loss of the BAC vector cassette from the RRV genome**

To examine the excision of BAC vector cassette from the BAC17 genome in mammalian cells, we retrofited the BAC vector cassette with a GFP cassette, and obtained a new RRV BAC clone designated BAC17GK. In addition to the BAC vector
cassette, BAC17GK also contained a GFP cassette at the same locus to facilitate the tracking of the BAC vector cassette. Upon reconstitution in rhesus fibroblasts, we only observed about 33% of foci that were GFP positive. IFA staining showed that all GFP-positive and -negative foci were gB-positive indicating that they were indeed caused by RRV infection (Fig. 3A). The percentage of the GFP-positive foci continued to decrease as the virus was passaged in culture. By passage 2, less than 5% of the foci remained GFP-positive. By passage 5, we no longer observed any GFP-positive foci albeit all the foci remained gB-positive (Fig. 3A). To confirm these results, we quantified the percentage of viral genomes containing the BAC vector cassette from virus preparations of different passages by qPCR. As shown in Fig. 3B, all the BAC genomes isolated from bacteria contained the BAC vector cassette (100%); however, the percentage of BAC vector cassette-positive genomes decreased rapidly as the viruses were passaged in culture. At passage 1, only 36% of the viruses contained the BAC vector cassette. At passage 2, only 3% of the viruses contained the BAC vector cassette. At passage 5, we could no longer detect any BAC vector cassette (Fig. 3B). These results indicated that the BAC/GFP cassettes were rapidly lost during the passage of the virus in rhesus fibroblasts.

Next, we directly isolated individual GFP-negative clones from passage 1 viruses. Since RRV is a slow growing virus, the ability to isolate viruses with the BAC vector cassette excised from the early passages could shorten the time of genetic manipulation. As controls, we also isolated GFP-positive clones. HindIII digestion showed that all the clones had similar restriction patterns as expected since the TR bands were >40 Kb and the addition of the BAC/GFP cassettes did not significantly
alter their migration patterns (Fig. 3C). Southern-blot hybridization with the BAC vector cassette probe detected a band of >40 Kb in all the GFP-positive clones but not in any of the GFP-negative clones (data not shown). We further examined the BAC vector locus-specific Kan cassette sequence by PCR. As expected, we detected the Kan sequence in GFP-positive clones as well as BAC17GK DNA isolated from bacteria and virions but not in GFP-negative clones and WT RRV (Fig. 3D). Because of the rapid spontaneous excision of the BAC vector cassette from the viral genome, we also detected weaker Kan sequence signals in the GFP-positive clones than the BAC17GK genome isolated from bacteria or virions. In contrast, several RRV-specific sequences including the most left and right sequences of the long unique region (LF and RF), Orf49 and RRTA were detected in all the RRV genomes (Fig. 3D). Finally, viruses from both GFP-positive and -negative clones caused similar extent of CPE in rhesus fibroblasts (Fig 3E). Together, these results indicated that the BAC vector cassette was no longer present in the BAC/GFP-negative RRV genome, and these BAC cassette-excised viruses remained infectious and genetically resembled the WT RRV.

**TR-mediated recombination contributes to the loss of the BAC vector cassette from the RRV genome**

To explore the possible mechanism mediating BAC vector cassette excision, we generated a BAC17 derivative BAC17GGK by replacing the chloramphenicol resistance gene with GFP and Kan cassettes. BAC17GGK has functional GPT and GFP expression cassettes in mammalian cells and a functional Kan cassette in bacteria. While BAC17 and its derivatives can be stably propagated bacteria DH10B (Fig. 2), they...
are prone to recombination in mammalian cells. It is known that DH10B are recombination-deficient; however, the mammalian cells are recombination-competent. Virions of BAC17GGK were produced by transfecting the viral DNA into rhesus fibroblasts. The recovered virions were then used to infect rhesus fibroblasts in the presence of MPA and xanthine. MPA inhibits the de novo synthesis of purines. However, GPT-expressing cells can overcome this defect in the presence of xanthine (15). Consequently, cells cultured in the pressure of MPA and xanthine should be enriched for viruses containing the BAC vector sequences, and thus should facilitate the recovery of viral genomes and any excised DNA molecules containing the BAC cassette in bacteria.

Two possible mechanisms could mediate the rapid loss of the BAC vector cassette from the RRV genome: (i) random cleavage of the concatemer at the TR regions during viral packaging; and (ii) TR-mediated homologous recombination. In the first scenario, if each viral genome is cleaved once at the TR when it is packaged, the loss of the BAC cassette should be at a rate of much less than 50% following each passage in cells. However, we observed much higher rate of BAC cassette loss (Fig. 3A and B). Thus, we concluded that this is not the predominant mechanism that mediates the BAC cassette loss. It is also possible that a viral genome is cleaved more than once at the TR region. In this case, the BAC cassette could be directly released from the viral genome as a linear fragment. Nevertheless, this DNA fragment cannot be recovered in bacteria unless it is ligated as a circular DNA. In the second scenario, the BAC cassette can be directly released from the viral genome as a circular DNA by TR-mediated recombination at any given time. This circular DNA can be directly propagated in bacteria.
bacteria without further ligation. To determine whether the loss of the BAC cassette is mediated by cleavage or homologous recombination, we infected rhesus fibroblasts with BAC17GGK virions and isolated total DNA from cells at 48 hpi. The total DNA was used to transform bacteria DH10B by heat shock to recover any circular DNA molecules that are less than 50 Kb. We observed large number of kanamycin-resistant colonies. We did not observe any increase in the number of kanamycin-resistant colonies upon ligation of the DNA with T4 DNA ligase before bacteria transformation. These results indicated that the BAC cassette-containing DNA molecules were circular, and thus were derived from homologous recombination but not cleavage. BamHI and EcoRI restriction analysis of the clones identified 10 patterns (Fig. 4A). Of 72 clones screened, they all fell into one of these patterns. Both BamHI and EcoRI have one site within the BAC vector sequence. Following digestion with BamHI and EcoRI, a 6.5 Kb fragment was released from BAC vector sequence while another band should be derived from the RRV sequence and part of the BAC vector sequence. Interestingly, the difference between any adjacent clones is about 1.8 Kb (Fig. 4A), which corresponds to size of the RRV TR unit (unpublished data). We further digested all the clones with BglII, which release the RRV TR unit. BglII has 4 sites in the BAC vector, which can release three fragments of 2.5, 2.1 and 1.9 Kb from the BAC vector (Fig. 4B). A 3.9 Kb fragment derived from the 2.1 Kb BAC vector partial sequence and almost one TR unit was also observed. Pattern 1 did not release any intact TR unit (1.8 Kb) because the majority of the only TR unit is present in the 3.9 Kb band. However, all the other patterns released intact TR unit following BglII digestion with the large size clones having more TR unit (Fig. 4B). Together, these results indicate that the loss of the BAC cassette is...
predominantly due to TR-mediated homologous recombination, and such recombination could occur between any two BAC cassette-flanking TR units. This mechanism explains the rapid loss of the BAC cassette following passage of the recombinant viruses in cells (Fig. 3A and B).

**BAC-excised RRV clones have the same growth characteristics as the WT RRV**

We compared the growth characteristics of the BAC vector cassette-excised viruses with the WT RRV and the GFP-positive virus BAC17GK. We observed similar growth characteristics for all the viruses examined in one step grow curve (Fig. 5). The titers of all the viruses increased from day 0 to day 3, reaching peak at day 3, after which they started to decrease (Fig. 5).

**Generation of a RRV mutant with RRTA deleted**

To demonstrate the feasibility of using the auto-excisable infectious BAC for genetic manipulation, we used BAC17GK to generate a RRV mutant with RRTA replaced with mRFP1. The procedure for generating the RRTA mutant was illustrated in Fig. 6A. We obtained the first recombination fragment consisting of an I-SceI recognition site, a Zeo cassette and a disrupted mRFP1 cassette by PCR amplification of pRSETB-mRFP1-Zin using primers RRTA-mRFP1F1 and RRTA-mRFP1R containing a 62 bp (68267-62328 nt) and a 60 bp (68081-68022 nt) of the RRV sequences (AF2010726), respectively. Upon the first homologous recombination we obtained an intermediate recombinant RRV BAC17GKΔRRTAmRFP1Z, in which the I-SceI recognition site and the Zeo cassette was flanked with two identical 53-bp DNA sequences from the mRFP1
(334-386 nt, AF506027). The second homologous recombination between these two identical 53-bp sequences excised the Zeo cassette, and generated a functional mRFP1. The resulting mutant BAC, in which the RRTA was replaced with the mRFP1, was designated as BAC17GKΔRRTAmRFP1. HindIII restriction digestion showed that the 2,963 bp RRTA band (65585 to 68547 nt, AF2010726) in the BAC17GK lane was shifted to a 1,928 bp band in the BAC17GKΔRRTAmRFP1 lane because of the replacement of a 1,707 bp RRTA fragment (66315 to 68021 nt, AF2010726) by a 672 bp mRFP1 fragment (4 to 675 nt, AF506027) (Fig. 6B). PCR amplification with primers SRRAF1 and SRRAR1 outside the RRTA region confirmed the size changes of the amplified products because of the replacement of the RRTA fragment by the mRFP1 fragment (Fig. 6C), which was further confirmed by DNA sequencing (Fig. 6D).

Previous studies have shown that RRTA has two exons (12, 17). The BAC17GKΔRRTAmRFP1 mutant had a replacement of RRTA in the second exon at aa 9 to aa 577 (AAK28330) with mRFP1 at aa 2 to aa 225 (AAM54544). Consistent with the results of other gammaherpesviruses including MHV-68, KSHV and EBV, for which RTA is an essential gene for viral lytic replication, we did not observe any CPE or foci formation upon reconstitution of BAC17GKΔRRTAmRFP1 in rhesus fibroblasts (data not shown). In order to rescue the mutant virus, we established a stable p3Flag-RRTA-Bsd rhesus fibroblast cell line expressing a 3xFlag RRTA (Fig. 6E). Upon reconstitution in the RRTA-expressing cells, BAC17GKΔRRTAmRFP1 caused CPE and formed both GFP- and RFP-positive foci. Similar to BAC17GK, the GFP-positive foci were rapidly lost with increasing passages in culture (data not shown). By passage 4, we no longer detected any GFP-positive foci (Fig. 6F). Several RFP-positive, GFP-negative clones
were isolated at passages 1 and 4. Further characterization of these BAC vector-
excised mutants is currently underway.

Discussion

Infectious BAC clones of herpesviruses have become powerful genetic tools for
studying the functions of genes encoded by these viruses. Using BAC clones, in
conjunction with the ET-recombination and transposon-mediated mutagenesis systems,
viral mutants can be easily generated in bacteria, and their phenotypes assessed in
mammalian cells (24). In spite of the advantage of the BAC technology for genetic
manipulation, the integration of a BAC vector cassette into the viral genome often
causes genetic and phenotypic alterations to the virus, and thus complicates the
characterization of the viral mutants (32). These issues arise due to the inserted extra
genetic materials that might surpass the tolerable size limit of the viral genome, causing
genomic instability and inefficient packaging of the viral genomes in the virions. The
extra cassette could also alter the expression of viral genes by direct disruption of the
viral genes or regulatory elements if the insertion site is not chosen appropriately, or by
influencing the transcriptional activities of the adjacent genes because of the addition of
extra transcriptional regulatory elements such as a CMV promoter required to drive a
marker cassette (38). Consequently, it is desirable to excise all the inserted cassettes
from the mutant viruses before any phenotypic characterizations. Herpesviruses have
either internal or TR regions containing multiple units of the repeat sequences that are
sensitive to homologous recombination. These repeat regions are intrinsic genetic
elements of the viruses that have coevolved with viruses and acquired the ability to
maintain their relative stability by possessing multiple identical repeat units. Thus, the internal or TR regions of herpesviruses are likely ideal for inserting the BAC vector cassette for genetic manipulation and its subsequent auto-excision. In this study, we have successfully obtained a RRV BAC with the BAC vector cassette inserted in the TR using a recently-developed transposon-mediated cloning strategy (37). We have shown that this RRV BAC is stable in bacteria upon serial passages, which is critical for reliable genetic manipulation in bacteria. Upon reconstitution in rhesus fibroblasts, we have shown that the BAC vector cassette is rapidly excised yielding viruses that genetically and phenotypically resemble the WT virus. We were able to obtain individual BAC-excised clones at as early as passage 1 immediately following reconstitution in rhesus fibroblasts. Thus, our results have shown that RRV TR can be used for inserting the BAC vector cassette, which can be subsequently excised from the viral genome. While possible, we have not shown that the internal repeat region can be used for similar application in this study due to the fact that the three RRV internal repeat regions are small (~1 Kb) (26), which had prevented us from obtaining a RRV clone with the BAC cassette inserted in anyone of these regions. Obviously, large size of the repeat regions not only help the selection of BAC clones with the cassette inserted in the repeat regions but also facilitate homologous recombination and the excision of the inserted cassettes. We expect that the efficiency of homologous recombination is much lower in the small internal repeat regions than the TR region, which would make the later region more attractive for genetic manipulation.

Other approaches have previously been developed to excise the BAC cassette from herpesviral genome. By inserting a fragment of identical viral sequences flanking
the BAC vector cassette, Wagner and colleagues were able to excise the cassette from
their MCMV BAC (32). While the BAC clone is stable in the recombination-deficient
bacteria, further mutagenesis could be complicated because of the presence of the
recombination sequence when the BAC genome is introduced into bacteria to generate
viral mutant by ET-recombination. Since ET-recombination system is commonly used
for BAC-based genetic manipulation, this instable property could render the approach
difficult to use. Furthermore, the excision process requires five serial passages of the
virus in cell culture for complete excision of the BAC vector followed by Southern-blot
hybridization for confirmation (32). Thus, it is a relatively time-consuming procedure. In
a second approach, Smith and colleagues have successfully adapted the Cre-loxP
system to excise the BAC cassette (27). However, this approach leaves a loxP site in
the viral genome, which could also cause phenotypic alterations to the virus (36).
Tischer and colleagues have developed a recombinant VZV containing an inverted
duplication of the viral genomic sequences in the mini-F replicon resulting in the
markerless excision of the vector sequences upon reconstitution in mammalian cells.
However, it is difficult to use this method to generate viral mutants with mutated
sequences adjacent to the BAC vector sequence. In contrast, our system allows the
generation of viral mutations in any genomic locations except those within the TR region.
To investigate the mechanism mediating BAC cassette loss, we generated a
BAC clone BAC17GGK containing a functional GPT cassette in mammalian cells, which
facilitate the enrichment of BAC17GGK-containing cells and the subsequent isolation of
the excised cassettes. While random cleavage of the concatemer at the TR regions
during viral packaging is a possible mechanism that might mediate BAC cassette loss,
our results indicate that this is unlikely the main mechanism. We observed much higher
rate of BAC cassette loss than the expected much less than 50% rate (Fig. 3A and B).
Furthermore, random cleavage of the concatemer at the TR regions should also
generate some viral genomes that are enriched for 2 or more copies of BAC cassette
per viral genome. However, we failed to observe the presence of such BAC genomes
following their recovery in bacteria (data not shown).
While both multiple cleavages at the same TR region and homologous
recombination can lead to the release the BAC cassette from the viral genome, our
results showed that almost all the recovered BAC cassette DNA molecules are circular
DNA, indicating that they were derived from homologous recombination. Additional
analysis showed the recovered DNA contained 1-10 TR unit, suggesting that the
recombination process could occur between any two TR units (Fig. 4B). Such flexibility
could explain the rapid loss of the BAC cassette upon passage in cells.
The retrofitting of a GFP cassette adjacent to the BAC vector cassette in the
auto-excisable RRV BAC allows the direct visualization of the cassettes-containing foci,
and thus greatly facilitates the selection of cassettes-excised RRV BAC clones. We
have demonstrated the feasibility of selecting cassettes-excised clones with both the
auto-excisable RRV BAC and the RRTA mutant BAC. Together, our results illustrate the
usefulness of this auto-excisable RRV BAC system for generating viral mutants and
delineating the functions of RRV genes.
Acknowledgements

We thank Dr. Ronald C. Desrosiers at New England Primate Research Center for providing the RRV strain RRV26-95, Dr. Woody Wright at the University of Texas Southwestern Medical Center for the pBABEhTERT retrovirus, Dr. Nikolaus Osterrieder at Cornell University for the pBAD-I-SceI plasmid, and Dr. Roger Tsien at the University of California at San Diego for the pRSETB-mRFP1 plasmid. This work was supported by grants from the National Institute of Health (CA132637, CA096512 and CA124332) to S.J.G. and (RR00163 and CA75922) S.W.W.

References


Figure Legends

Fig. 1. Genetic analysis of recombinant RRV BAC17. (A) HindIII or EcoRI restriction analysis of BAC17 DNA isolated from bacteria and virion DNA of WT RRV. (B-C) Southern-blot hybridization with probes of BAC vector pHAI (B) and RRTA (C). M stands for 1 Kb plus DNA marker.

Fig. 2. Recombinant RRV BAC17 is stable in bacteria. (A) Restriction analysis of BAC17 following multiple passages in bacteria. DNA isolated from different passages of BAC17 were analyzed by HindIII digestion. (B-C) Southern-blot hybridization with probes of BAC vector pHAI (B) and RRTA (C). M stands for 1 Kb plus DNA marker.

Fig. 3. Rapid excision of BAC vector cassette from the RRV17GK genome in rhesus fibroblasts. (A) Rapid loss GFP-positive foci following serial passages in rhesus fibroblasts. Total RRV foci were detected by staining for RRV gB while GFP-positive foci were identified by directly visualizing GFP. Cell nuclei were revealed by DAPI staining. (B) Rapid loss of BAC cassette sequence from the RRV17GK genome. The percentage of BAC-positive RRV17GK genomes were quantified by determining the BAC cassette copy numbers and the RRV genomic copy numbers in DNA from different passages of viruses by qPCR for the Kan sequence, an indicator for the BAC cassette, and the RRTA sequence, an indicator for the RRV genome, respectively. For each passage, the virus was inoculated in three wells. Results were the averages from three wells. (C) BAC clones with the BAC vector from bacteria was set as 100% because all the viral genomes from bacteria contained the BAC vector cassettes. (C) BAC clones with the BAC vector
cassette excised have restriction pattern similar to that of WT RRV. DNA from GFP-negative clones (clones 1 and 2) and GFP-positive clones (clones 3 and 4), BAC17GK DNA from bacteria and virions, and DNA from WT RRV virions were subjected to HindIII restriction analysis. (D) GFP-negative clones no longer contain the BAC vector cassette. GFP-negative clones (clones 1 and 2) and WT RRV were negative for the Kan sequence while GFP-positive clones (clones 3 and 4), and bacteria and virion BAC17GK were positive for the Kan sequence. GFP-positive clones had less Kan signal because of the spontaneous excision of the BAC vector cassette. All the RRV genomes were positive for the RRV-specific sequences including the most left and right sequences of the long unique region (LF and RF), Orf49 and RRTA. (E) BAC clones with the BAC vector cassette excised remained infectious. Both GFP-negative clone (clone 1) and GFP-positive clone (clones 3) caused foci formation in rhesus fibroblasts.

Fig. 4. Analysis of excised BAC cassette clones recovered from recombinant RRV BAC17GGK infected rhesus fibroblasts. (A) EcoRI and BamHI dual digestion. Total DNA isolated from BAC17GGK infected rhesus fibroblasts were transformed into bacteria by the heat shock method. DNA preparations from bacteria clones were analyzed by EcoRI and BamHI dual digestion. All the isolated clones belong to one of the 10 identified patterns (lanes 1-10). (B) BgIII digestion. The same DNA preparations described in (A) were subjected to BgIII digestion. M stands for 1 Kb plus DNA marker.

Fig. 5. Growth characteristics of recombinant RRV17GK and its BAC vector cassette excised clones. RRV17GK and its BAC vector cassette excised clones had growth
curves similar to WT RRV in one step growth curve. Rhesus fibroblasts in 6-well plates were infected with different viruses at 2.5 TCID$_{50}$ per cell. Supernatants from the infected rhesus fibroblasts were collected at different day post-infection for titer determination to obtain the growth curves.

Fig. 6. Generation of a RRV mutant with RRTA deleted using the auto-excisable RRV BAC. (A) A schematic illustration showing the strategy for generating a RRV mutant with RRTA deleted. (B) HindIII restriction analysis of the RRTA mutant. (C) PCR confirmation of the replacement of RRTA by mRFP1 using primers outside the RRTA region. (D) DNA sequencing to confirm the replacement of RRTA by mRFP1. (E) Establishment of a stable rhesus fibroblast cell line expressing RRTA. 3xFlag RRTA was detected with an anti-Flag antibody (red). Nuclei were identified by DAPI staining. (F) Auto-excision of BAC vector cassette from the RRTA mutant. Rapid loss GFP-positive foci following serial passages in rhesus fibroblasts. All the foci were RFP-positive because of the replacement of RRTA by the mRFP1 cassette.
Figure 1

A

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B

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C

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Kb

- >30
- 11
- 6
- 7.25
- 2.96

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Figure 2

A

B

cpB probe

RRTA probe

C

Kb

>30

2.96

pHA probe

RRTA probe
Figure 3

DAPI                gB                 GFP             Merge

BAC cassette-containing viruses (%)

Passage

M          WT RRV
Cell DNA
BAC17GK
/bacteria
BAC17GK
/virion
Clone 1
Clone 2
Clone 3
Clone 4

M          Kan
Orf49
RRTA
LF
RF

bp

Clone 3                 Clone 1
E
D
C
B
Figure 4

A

B

EcoRI + BamHI

BgIII
Figure 5

Viral titer (lgTCID<sub>50</sub>/0.05 ml)

Day post-infection

WT RRV
BAC17GK
Clone 1
Clone 2