Functional Characterization of Kaposi's Sarcoma-Associated Herpesvirus Open Reading Frame K8 by Bacterial Artificial Chromosome-Based Mutagenesis^{∇†}

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The open reading frame K8 of Kaposi's sarcoma-associated herpesvirus (KSHV) encodes a basic leucine zipper (bZip) protein that binds to the origin of viral DNA replication and is an integral component of viral lytic DNA replication complex. Moreover, K8 physically interacts with replication and transcription activator (RTA) and represses its transactivation activity on several viral promoters. To investigate the role of this protein in viral life cycle, we constructed two K8-null recombinant mutant viruses (BAC-ΔK8 and BAC-stopK8) by using a bacterial artificial chromosome (BAC) system. Latent viral infection can be reconstituted in 293T and BJAB cells with wild-type and the K8-null recombinant viruses by introducing the cloned viral genomes into the cells. When the cells carrying these viruses were induced with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and sodium butyrate, no significant difference was seen in overall viral gene expression between wild-type and K8-null viruses, with lytic DNA replication still active in the latter. However, 293T cells harboring K8-null mutant viruses, either BAC-ΔK8 or BAC-stopK8, displayed lower copy numbers of latent KSHV genome in comparison with wild-type viruses. Furthermore, although K8 deficiency appeared to not affect infectivity when K8-null viruses were used to infect 293T, primary human microvascular dermal endothelial and human foreskin fibroblast cells, they exhibited much lower viral genome copy numbers in all types of cell compared to wild-type viruses. Taken together, these data suggest a possible role of K8 in abortive lytic DNA replication occurring in early stages of *de novo* infection or in the maintenance of latent viral genomes.

Kaposi's sarcoma-associated herpesvirus (KSHV), also referred to as the human herpesvirus 8 (HHV-8), is a human gammaherpesvirus and the etiological agent of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (7). Similar to other herpesviruses, KSHV has two distinct life cycles, namely, the latent and lytic. Unique among human tumor viruses, the lytic cycle of KSHV is crucial for its tumorigenesis. Studies have shown that the KSHV productive replication cycle directly contributes to viral tumorigenesis by spreading viruses to target cells and providing paracrine regulation for KS development (3). A study by Grundhoff and Ganem (9) suggests a new role of lytic replication in sustaining the population of latently infected cells that otherwise would be quickly lost by segregation of latent viral episomes as spindle cells divide. Thus, KSHV lytic replication and constant primary infection to fresh cells are crucial for both viral tumorigenicity and pathogenesis.

The lytic life cycle of a herpesvirus is initiated and controlled by a small number of regulatory proteins encoded by viral immediate-early (IE) genes. Several KSHV IE genes have been identified, including open reading frame 50 (ORF50), ORF45, and K8 (32). Among them, ORF50 encodes a transcriptional activator, designated RTA (for replication and

transcription activator), which is able to switch on viral lytic replication when expressed in latently infected B lymphocytes (19, 23). ORF45, on the other hand, was found to interact with cellular interferon regulatory factor 7 (IRF-7), inhibiting its activation in response to virus infection (33, 34). ORF45 was also reported to be involved in activation of RSK-ERK signaling that is required for KSHV lytic replication (14). The ORF K8 of KSHV encodes a bZip protein, termed the K8 protein or replication-associated protein (RAP) or K-bZip (18, 32). During viral reactivation, the K8 gene gets expressed in both the IE and delayed-early stages, driven by two distinct promoters (24). K8 mRNA is highly spliced and contains three coding exons and one untranslated exon. Alternative splicing yields three splice variants, namely, K8 α , K8 β , and K8 γ , with K8 α representing the predominant isoform encoding a 237-amino-acid protein containing a basic and leucine zipper domain (18, 32).

K8 is the positional and structural analogue of Epstein-Barr virus (EBV) Zta (also called ZEBRA or EB1). Zta is encoded by an EBV IE gene BZLF1 and is associated with three important functions: (i) Zta is a transcriptional activator that activates the viral lytic gene expression cascade and initiates the switch of EBV from latency to lytic life cycle (21); (ii) Zta causes host cell cycle arrest at G₀/G₁ phase (4); and (iii) Zta binds to EBV *ori-Lyt*, and this binding is essential for viral lytic DNA replication (5). On similar lines, efforts have been undertaken to determine whether KSHV K8 also possesses roles in gene transcription similar to its EBV counterpart. Although no transcriptional activation was found associated with K8, it was found to interact with RTA (ORF50), thereby repressing the RTA-mediated transcription of viral delayed-early genes

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(10, 16). In addition, K8 was reported to inhibit the activity of CDK2 that plays a pivotal role in cell cycle progression. Inhibition of CDK2 activity by K8 was shown to result in a prolonged G₁ phase with concomitant induction of p21, P27, and C/EBP α (10, 29).

Interestingly, K8 was found to bind to the origin of lytic viral DNA replication (*ori-Lyt*) of KSHV (17). Further investigations have mapped the K8 binding region to a cluster of C/EBP binding motifs within the *ori-Lyt*. Substitution mutagenesis experiments have shown the requirement of these C/EBP palindromes toward binding of K8 protein in *ori-Lyt* DNA and for DNA replication (25). Direct evidence as to the essentiality of K8 in KSHV lytic DNA replication came from findings of a transient-transfection assay, which showed that KSHV lytic origin (*ori-Lyt*)-dependent DNA replication requires six core replication proteins, along with K8 and RTA; omission of K8 in this assay abolished the DNA replication (1).

From the aforementioned observations, it becomes evident that K8 is a multifunctional protein. However, the mechanisms behind the activities of K8 still remain elusive, with most of the data regarding K8 functions obtained only from transient assays. Thus, to study the role of K8 in the context of the actual viral life cycle, we generated K8-null recombinant viruses with bacterial artificial chromosome (BAC)-based recombineering techniques (15). Using the recombinant viruses, we investigated the role of K8 in the viral lytic DNA replication. To our surprise, it was revealed that K8 is not absolutely required for KSHV viral lytic DNA replication and viral propagation. More importantly, a possible role of K8 in viral DNA replication in the early stage of *de novo* infection or during maintenance of latent viral genomes was implicated.

MATERIALS AND METHODS

Cells and *Escherichia coli* strains. BJAB cells, virus-free lymphoblastoid B cells, were cultured in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS). Human embryonic kidney (HEK) 293T cells were obtained from ATCC. Human foreskin fibroblasts (HFF) (HFF2441) were kindly provided by Meenhard Herlyn at the Wistar Institute. Both 293T and HFF cells were cultured in Dulbecco modified Eagle medium supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics. Human dermal microvascular endothelial (HMVEC-d) cells (CC-2543) were purchased from Lonza Walkersville, Inc., and maintained in endothelial basal medium 2 in the presence of necessary growth factors. All cultures contained penicillin-streptomycin (50 U/ml), amphotericin B (1.25 μ g/ml [Fungizone]), and sodium desoxycholate (1.25 μ g/ml). *E. coli* strain EL350 was obtained from Neal Copeland and Nancy Jenkins at the National Cancer Institute, Frederick, MD (15). BAC36, which carries the entire KSHV genome, was obtained from Shou-Jiang Gao at the University of Texas at San Antonio and has been described previously (30).

Genetic manipulation of KSHV BAC genome. Mutagenesis of BAC36 was performed by using a recombineering system (<http://recombineering.ncicrf.gov>). BAC36 was first introduced into EL350 by electroporation. The EL350 strain contains a defective prophage that harbors the recombination genes *exo*, *beta*, and *gam* under tight control of a temperature-sensitive c1857 repressor. Recombination functions can be supplied transiently by transfer of the culture to 42°C for 15 min (15). To generate a K8 deletion mutant, we replaced the K8 coding sequence of BAC36 with a kanamycin (Kan)/SacB cassette by homologous recombination. The Kan/SacB cassette was amplified from plasmid pBS-Kan/SacB by PCR with primers K8-Kan/SacB-5' (5'-TTCCTCCGTTGTCGACTAT AACCTGGCGTGTAACGCTGTAACCTGCCAAGcatgacgtccacatatac-3') and K8-Kan/SacB-3' (5'-TACCACACAAAGTCTGGCATGTTCTCCCGGACGACGGCACAACCAAGCTctaccgcacagatgcgaagg-3'). Each primer contains 21 nucleotides (nt) homologous to the antibiotic resistance cassette Kan/SacB at its 3' end (lower case) and 50 nt homologous to the first 50 nt before the start and following the stop codons of K8 at the 5' end (upper case). These two primers were used to amplify the Kan/SacB cassette, which contains the kanamycin

resistance gene and SacB, by PCR. PCR was carried out at 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min for 30 cycles with Roche Expand high-fidelity *Taq* polymerase. The PCR product was treated with DpnI to remove the plasmid template. The digested product was gel purified with a QIAEX II gel extraction kit (Qiagen). The purified PCR fragment was electroporated into BAC36-containing EL350 cells that had been induced at 42°C for 15 min. The parameters for electroporation were set at 1.75 kV, 276 Ω , and 50 μ F in a 0.1-mm cuvette (BTX). The recombinant clones were selected at 32°C on LB plates containing 12.5 μ g of chloramphenicol and 50 μ g of kanamycin per ml and then characterized by PCR and Southern blot analyses. The sensitivity to sucrose (Suc) was tested by plating the same amount of overnight culture on plates containing LB plus Kan and on plates containing LB plus 7% Suc. The resultant BAC was designated BAC- Δ K8.

To make a revertant mutant, we replaced the Kan/SacB cassette in BAC- Δ K8 with a wild-type K8 sequence by a homologous recombination strategy similar to that described above. The wild-type K8 sequence was amplified by PCR, with GB22 cosmid DNA as a template and the oligonucleotides K8-revert5' (5'-TTCCTCCGTTGTCGACTATAACCTGGCGTGTAACGCTGTAACCTGCCAAATGCCAGAAATGAAGGACATACCTACTAAGAGTTCCTCCCGGGAACGGACAATTC-3') and K8-revert3' (5'-TACCACACAAAGTCTGGCATGTTCTCCCGGACGACGCGGACAACCAAGCT-3') as primers. GB22 cosmid DNA was obtained from Ren Sun and has been described earlier (32). In addition, a mutant containing a premature stop codon was constructed. The Kan/SacB cassette in BAC- Δ K8 was replaced with a K8 sequence-of-stop-codon mutant, which was generated by PCR with the primers K8-stops5' (5'-TTCCTCCGTTGTCGACTATAACCTGGCGTGTAACGCTGTAACCTGCCAAATGCCAGAAATGAAGGACATACCTAGTTCCTCCCGGGAACGGACAATTC-3') and K8-revert3'. The lowercase letters "tag" in the forward primer represent a single point mutation introduced at the tenth codon and replaces the AAG codon with a stop codon.

About 200 ng of gel-purified PCR fragments was electroporated into induced EL350 cells carrying BAC- Δ K8. The transformants were selected at 32°C on LB plates containing 12.5 μ g of chloramphenicol/ml and 7% sucrose. Because of frequent undesired recombination, many of the Kan^r/Suc^r colonies did not have the expected recombination. To identify the clones that did, we performed *in situ* colony hybridization with the wild-type K8 coding sequence as a probe. The positive clones were expanded, and the BAC DNAs were extracted and analyzed by PCR and Southern blot analyses. All BAC clones were also sequenced with primer K8up (5'-ATAGCTCATTTTCTCAGCAATC-3'). The BAC DNAs with the proper recombination were prepared from overnight cultures with a large-construct kit (Qiagen).

Reconstitution of recombinant viruses. The freshly prepared BAC DNAs were introduced into 293T cells by means of a Qiagen Effectene transfection kit. In brief, 1 μ g of BAC DNA was used to transfect 293T cells grown to 40 to 60% confluence in 60-mm dishes. At 2 days after transfection, the cells were examined by fluorescence microscopy and then subcultured in T150 flasks with fresh medium containing 200 μ g of hygromycin/ml. When the colonies were visible (usually 10 to 14 days after transfection), they were dislodged and seeded into a new T150 flask. When the monolayer reached 80 to 90% confluence, cells from each flask were split into three new T150 flasks. Three days later, the cells were split again and induced with 20 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)/ml and 0.3 mM sodium butyrate. Usually, 20 or more T150 flasks (150 cm²) of cells were induced for 4 to 5 days.

For reconstitution of recombinant viruses in B lymphocytes, freshly prepared BAC DNAs were introduced into BJAB cells by electroporation. Transformed cells were cultured in RPMI 1640 medium and selected with 200 μ g of hygromycin/ml for 3 to 4 weeks. Lytic life cycle of the virus was induced by adding 20 ng of TPA/ml and 0.3 mM sodium butyrate to the medium. The induced culture media were collected and filtered through 0.45- μ m-pore-size filters. The virions were then pelleted by ultracentrifugation on a 25% sucrose cushion at 100,000 $\times$ g for 1 h with a Beckman SW28 rotor. The pellets were dissolved in 1% of the original volume of 1 $\times$ phosphate-buffered saline (PBS) or Dulbecco modified Eagle medium and stored at -80°C.

Preparation and quantification of genomic and virion DNAs. Total DNAs were prepared from viral stocks or cells with a DNeasy tissue kit (Qiagen). Monolayers of infected cells in 60-mm dishes were treated with trypsin, washed, and resuspended in 200 μ l of 1 $\times$ PBS. Total DNA was prepared according to the manufacturer's instructions. For the preparation of DNA from intact virions, 200 μ l of virus stocks was pretreated with 10 μ l of Turbo DNase I (Ambion) for 1 h at 37°C. The reaction was stopped by addition of EDTA, followed by heat inactivation at 70°C. Then, 20 μ l of proteinase K solution and 200 μ l of buffer AL from the DNeasy kit (Qiagen) were added. The mixture was kept at 56°C for 15 min and then extracted with phenol-chloroform. The DNA was ethanol precipi-

itated with glycogen, and the DNA pellet was dissolved in 40 μ l of Tris-EDTA buffer.

Copy numbers of KSHV genomic DNA in viral stocks and in the infected cells were estimated by real-time DNA PCR. Briefly, DNA was prepared with a DNeasy tissue kit (Qiagen), and DNA samples were subjected to real-time DNA PCR with a Roche LightCycler and a LightCycler FastStart DNA Master^{Plus} SYBR green kit. Viral DNA copy numbers were calculated from external standards of known concentrations of BAC36 DNA. The primers ORF73-LCN (5'-CGCGAATACCGCTATGTACTCA-3') and ORF73-LCC (5'-GGAACGCGCCTCATACGA-3') have been previously described by Krishnan et al. (13).

Western blotting. Cells were washed with 1 $\times$ PBS and lysed with cell lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate [Na₃VO₄], 20 mM sodium pyrophosphate, 100 mM sodium fluoride, 10% glycerol, 1 mM EDTA, 5 μ g of aprotinin/ml, 5 μ g of leupeptin/ml, 5 mM benzamide, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were homogenized and centrifuged at 13,000 rpm for 5 min at 4°C. Portions (50 mg) of whole-cell extracts was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in 5% dried milk in 1 $\times$ PBS plus 0.2% Tween 20 and then incubated with diluted primary antibodies for 2 h at room temperature or overnight at 4°C. Anti-rabbit or anti-mouse immunoglobulin G antibodies conjugated to horseradish peroxidase (Amersham) were used as the secondary antibodies. An enhanced chemiluminescence system (Amersham) was used for detection of antibody-antigen complexes.

Quantitative reverse transcription-PCR. Cells carrying the wild type or K8-null mutants were induced with 20 ng of TPA/ml and 0.3 mM sodium butyrate for indicated periods. Cells were lysed with TRIzol reagent (Invitrogen) and total RNAs isolated according to the manufacturer's protocol. Residual DNA contamination was eliminated by subsequent treatment with Turbo DNase I (Ambion). cDNA was generated from total RNA with the SuperScript first-strand synthesis system (Invitrogen), using priming with random hexamers. The cDNA thus obtained was analyzed by SYBR-green based real-time PCR approach with primers specific to viral mRNA indicated. The expression levels obtained for each gene with wild-type (BAC36) and the K8-null mutant (BAC- Δ K8 and BAC-stopK8) viruses were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The fold change for a particular gene of interest in response to TPA/butyrate induction was subsequently calculated by comparing the normalized value of that gene prior to and after induction.

Gardella gel assay. Approximately 5×10^5 293T or BJAB cells carrying BAC36 and K8-null mutant viruses, respectively, were collected 3 days after induction for lytic KSHV replication. The cells were pelleted and resuspended in 100 μ l of sample buffer A, containing 15% Ficoll, 40 μ g of RNase A/ml, and 0.01% bromophenol blue in 1 $\times$ Tris-borate-EDTA and then loaded into 3-by-10-mm slots of a vertical 0.8% agarose gel (160 mm long by 200 mm wide by 3 mm thick). A total of 100 μ l of lysis buffer, containing 5% Ficoll, 1% SDS, 1 mg of pronase/ml, and 0.05% xylene cyanol was carefully overlaid on the cell suspension, and the gel was electrophoresed at 0.8 V/cm for 3 h and then at 5 V/cm for 12 h at 4°C with 1 $\times$ Tris-borate-EDTA running buffer. After electrophoresis, the gel was treated with 0.25 M HCl for 15 min and then with 0.5 M NaOH-1.5 M NaCl and finally neutralized with 0.5 M Tris-HCl (pH 7.5)-1.5 M NaCl. The DNA was transferred with 10 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) onto a Nytran membrane and hybridized with a ³²P-labeled KSHV-specific probe.

Transient-transfection DNA replication assay. Plasmid pOri-A was constructed by cloning an EcoRI-PstI fragment (nt 22409 to 26491) of KSHV DNA in pBluescript at the EcoRI/PstI site as described previously. pCR3.1-ORF50 and pCR3.1-K8 α were constructed by cloning the cDNA sequences of ORF50 and K8 α coding region into pCR3.1 vector (Invitrogen). These three constructs are described in detail in Lin et al. (17).

To assay the lytic DNA replication of BAC-cloned KSHV genomes and mutants, 293T cells were seeded into 60-mm dishes at 24 h prior to transfection. Monolayer cells were transfected with BAC36 or K8-null mutant (BAC- Δ K8 and BAC-stopK8) DNAs (6 μ g), pCR3.1-ORF50 (1 μ g), and pCR3.1-K8 α (1 μ g) as indicated using Lipofectamine 2000 (Invitrogen).

To assay *ori-Lyt*-dependent DNA replication, pooled hygromycin-resistant 293T monolayer cells that carry BAC-36 and K8-null mutant genomes were transfected with the plasmids pOri-A (2 μ g), pCR3.1-ORF50 (1 μ g), and pCR-3.1-K8 α (1 μ g) as indicated using Lipofectamine 2000.

At 72 h posttransfection, extrachromosomal DNAs were prepared from cells by using the Hirt DNA extraction method as follows. Cells were lysed in 700 μ l of lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM EDTA, and 0.6% SDS). Chromosomal DNA was precipitated at 4°C overnight by adding 5 M NaCl to a final concentration of 0.85 M. Cell lysates were centrifuged at 4°C at 14,000 rpm

for 30 min. The supernatant containing extrachromosomal DNA was subjected to phenol-chloroform extraction, followed by ethanol precipitation. The DNA was treated with RNase A at 25°C for 30 min and then with proteinase K at 50°C for 30 min. Then, 5 μ g of DNA was digested with KpnI/SacI or KpnI/SacI/DpnI (New England Biolabs). The DNAs were separated by electrophoresis on 1% agarose gels and transferred onto GeneScreen membranes (Perkin-Elmer, Boston, MA). The Southern blots were hybridized with a ³²P-labeled KSHV specific probe (for detection of newly replicated BAC DNAs) and pBluescript plasmid (for detection of newly replicated *ori-Lyt* plasmid) in 5 $\times$ SSC, 2 $\times$ Denhardt solution, 1% SDS, and 50 mg of denatured salmon sperm DNA/ml at 68°C.

Infection of 293T, HMVEC-d, and HFF cells with recombinant viruses. Cells plated in 24-well plates were incubated with concentrated virus and spun at 2,500 rpm for 1 h at room temperature. The inocula were then removed and replaced with fresh media with 5% FBS. The following day, the media were replaced with fresh medium containing 1% FBS. Green fluorescent protein (GFP) expression was used to monitor infection 2 days after infection. At the indicated time points postinfection, the total DNA was isolated from infected cells, and viral genomic DNAs were quantified by a real-time PCR with primers specific for KSHV ORF73. Viral DNA copy numbers were calculated as described above.

RESULTS

Generation of K8-null recombinant KSHV. To investigate the roles of K8 in the viral life cycle, we attempted to construct K8-null KSHV recombinant viruses. A BAC-cloned KSHV (BAC36) constructed and reported previously (30) was used as a template. The BAC36 carries the full KSHV genome, and infectious KSHV can be reconstituted from it by transfection of BAC36 DNAs into 293 cells (30). BAC36 contains GFP and hygromycin resistance gene markers, allowing easy detection of eukaryotic cells containing the KSHV genomes. To generate a K8-null mutant virus, we used a two-step replacement procedure. In the first step, the K8 coding sequence in BAC36 was replaced with a Kan/SacB cassette through recombineering technology (recombination-mediated genetic engineering). Briefly, a bacterial double selection cassette Kan/SacB flanked by sequences homologous to the first 50 nt before the initiation codon of K8 and the last 50 nt next to the stop codon of K8 at two ends was synthesized by PCR and transformed into *E. coli* EL350 cells carrying BAC36. Induction of recombination activity in the EL350 cells at 42°C resulted in the replacement of K8 by the Kan/SacB cassette. Transformants were selected by kanamycin resistance (Kan⁺). DNAs were isolated from kanamycin-resistant colonies, digested with restriction enzymes, and analyzed on 0.8% agarose gels. Digestion of the wild-type BAC36 DNA with XhoI generated a fragment of 12.5 kb at the ORF K8 locus. Replacement of K8 coding sequence with the Kan/SacB cassette shifted the fragment size to 14.6 kb (Fig. 1A and B). Sequencing analysis of the mutant viral DNA confirmed the successful replacement of the K8 coding sequence with that of the Kan/SacB cassette (Fig. 1C), and the resulting mutant was designated BAC- Δ K8.

In the second step, we generated a rescued revertant virus and a stop codon mutant. A PCR fragment of wild-type K8 sequence was introduced into BAC- Δ K8-bearing EL350 cells. A desired recombination was expected to replace the Kan/SacB cassette in BAC- Δ K8 with a wild-type K8 sequence. The Kan/SacB cassette has a double-selection property permitting both positive and negative selection. SacB, originally from *Bacillus subtilis*, encodes an enzyme that converts sucrose to levan, which is toxic to bacteria and causes cell death (8). Bacteria that express SacB are killed in medium containing sucrose but grow normally in medium without it. The revertant

FIG. 1. Construction and analyses of K8-null mutant BACs. (A) Schematic diagrams of the structures of K8 in the wild-type or mutant BACs. The nucleotide sequences refer to GenBank accession number U75698. (B) Electrophoretic analysis of K8-null mutant viral genomes. Wild-type (BAC36), BAC-ΔK8, and BAC-stopK8 DNAs were digested with XhoI, resolved on a 0.8% agarose gel, and stained with ethidium bromide. The 12.5- and 14.6-kb fragments from BAC36 and BAC-ΔK8, respectively, affected by the insertion are indicated by red arrows. (C) Sequences of the wild-type and K8-null mutant BAC DNAs at the K8 locus. The BAC DNAs were purified with a Qiagen large-construct kit and subjected to automated DNA sequencing. The sequence chromatograms of these clones are shown. The single point mutation from AAG to stop codon TAG in BAC-stopK8 is boxed.

mutants can thus be screened for both sucrose-resistant and kanamycin-sensitive phenotypes. Selected clones were verified by restriction enzyme digestion and Southern blot analysis. The clones that displayed the expected pattern was designated BAC-revK8. Similarly, a mutant that carries a premature stop codon was also constructed by the same strategy and designated BAC-stopK8. The BAC-stopK8 clones were also verified by restriction enzyme digestion (Fig. 1B), Southern blot analysis (data not shown), and sequencing analysis (Fig. 1C). The sequencing data confirmed that BAC-stopK8 carries an AAG to TAG single codon mutation at the tenth codon (Fig. 1C).

K8 deficiency has no significant effect on the KSHV gene expression. To reconstitute recombinant viruses, we transfected 293T cells with BAC36 and K8-null mutant BAC DNAs.

The presence of hygromycin resistance and GFP markers in the BAC36 genomes enabled us to monitor the transfection efficiencies and to enrich transfected cells. Since KSHV typically establishes latent infection by default, the cells harboring the BAC genomes can be enriched and maintained in medium containing hygromycin (30). When almost 100% of cells became positive for GFP expression after selection, the cells were treated with TPA/*n*-butyrate to induce viral lytic replication. Whole-cell lysates were prepared from induced and uninduced cells and analyzed for the expression of viral genes of different kinetic categories by Western blot analysis with antibodies against various KSHV gene products. K8 was not detected in 293T cells carrying BAC-ΔK8 and BAC-stopK8 cells, whereas it could be detected easily in 293T BAC36 cells (Fig. 2) and

FIG. 2. Comparison of levels of viral gene expression of BAC36, BAC-ΔK8, and BAC-stopK8 in 293T cells. The pooled hygromycin-resistant 293T cells carrying BAC36, BAC-ΔK8, and BAC-stopK8 were induced with TPA and sodium butyrate for 0, 2 (2d), and 3 days (3d). The induced cells were lysed, and the whole-cell lysates were immunoblotted with the indicated antibodies against KSHV viral proteins of different kinetic categories.

293T BAC-revK8 cells (data not shown). The Western analyses detected no significant difference between wild-type KSHV (BAC36 and BAC-revK8) and K8-null viruses (BAC-ΔK8, BAC-stopK8) in the expression of latency-associated nuclear

antigen (LANA), ORF50 (RTA), ORF45, ORF57, ORF59 (polymerase processivity factor [PPF]), ORF64, and K8.1. This suggests that the absence of K8 expression in a mutant KSHV appears to have no effect on latent (LANA), immediate-early (RTA and ORF45), delayed-early (ORF57 and ORF59), and late (ORF64 and K8.1) gene expression (Fig. 2).

The mRNA levels of the recombinant viruses were also analyzed at 24 and 48 h postinduction using a quantitative real-time PCR. The results in general were consistent with those of the Western blots except that LANA mRNA was found to increase by 6.5- and 10.3-fold at 24 and 48 h postinduction in the wild-type KSHV (see Fig. S1 in the supplemental material), although no obvious increase was seen in the protein level, as detected by a Western blot (Fig. 2). Similar increase in LANA mRNA level upon the induction was also observed with K8-null KSHV. This increase we believe could be due to induction of the LANA lytically inducible promoter (20), though its biological significance remains elusive. It has been earlier reported that ORF57 accumulation (at a high multiplicity of infection [MOI]) could be 1,000-fold higher in K8-null cells than in wild-type-carrying cells under conditions where RTA was overexpressed (12). Our results, however, indicated that at least in TPA-butyrate induced cells, no significant increase in ORF57 expression was observed in K8-null virus carrying cells compared to cells carrying wild-type KSHV.

Viral lytic DNA replication appears not to be affected by K8 deficiency. K8 protein was found to bind to *ori-Lyt* and function as a component in viral replication complexes (17, 25, 27, 28). Direct evidence pertaining to the critical role of K8 in KSHV lytic DNA replication was obtained from a transient-transfection assay, which showed that KSHV *ori-Lyt*-dependent DNA replication requires six viral core replication proteins along with additional presence of K8 and RTA; omission of K8 in this assay was shown to abolish the DNA replication (1). However, most of the data regarding K8 function in KSHV DNA replication were obtained in transient assays and not in the context of the virus. This prompted us to use K8-null recombinant viruses to examine the role of K8 in viral lytic DNA replication. First, we carried out a Gardella gel assay on 293T cells harboring wild-type or K8-null recombinant viruses to assess the impact of K8 deficiency on viral lytic DNA replication. This assay uses a native vertical agarose gel electrophoresis that can distinguish between linear viral DNA, presumably the result of viral DNA replication, and circular KSHV DNA, representing the episomal version of the viral DNA (6). After TPA-butyrate induction, dramatic increases of the replicating (linear) form of viral DNA was seen with wild-type BAC36. Viral DNA replication was also detected in TPA induced 293T-BAC-ΔK8 and 293T-BAC-stopK8 monolayers although the levels were lower (Fig. 3, the quantitative analysis is shown in Fig. S2 in the supplemental material). These data suggest that viral lytic DNA replication is still active despite the absence of K8.

To further analyze the effect of K8 deficiency on KSHV lytic DNA replication, we examined the DNA replication of BAC cloned KSHV genomes (bacmids) in cells. BAC36 and K8-null mutants (BAC-ΔK8 and BAC-stopK8) were introduced into 293T cells by transfection along with an RTA expressing vector. Newly replicated DNA was distinguished from input bacmid by DpnI restriction digestion, which selectively cleaves

FIG. 3. Gardella gel analysis of viral lytic DNA replication. The pooled hygromycin-resistant 293T and BJAB cells were induced with TPA and sodium butyrate for 3 days. Approximately 5×10^5 of TPA-induced (+) and uninduced (-) BAC-containing 293T cells (A) and BJAB cells (B) were loaded onto a 0.8% vertical agarose gel. Samples were overlaid with lysis buffer and electrophoresed initially at 0.8 V/cm for 3 h and then at 4.5 V/cm for 12 h at 4°C. Gels were subsequently transferred onto nylon membranes and probed with a ^{32}P -labeled DNA fragment of the LANA coding sequence. The positions corresponding to the circular DNA form and the linear replicating form of KSHV are indicated.

input DNAs that have been methylated in *dam*⁺ *E. coli* without affecting DNA that has replicated at least once in eukaryotic cells. In other words, newly replicated bacmid DNA in cells is resistant to DpnI digestion and can be detected in Southern analysis. As shown in Fig. 4, both K8-null mutants were able to replicate in the presence of RTA. Ectopic expression of K8 did not enhance the replication. A phosphorimaging analysis of the DpnI assay is shown as supplementary data (see Fig. S3 in the supplemental material).

Although the experiments described above suggested that K8-null viruses can replicate in the lytic cycle, we were not sure at this point whether the viral DNA replication was *ori-Lyt* dependent. To address this question, we tested whether a plasmid carrying a KSHV *ori-Lyt* sequence can be replicated in the cells harboring the K8-null mutant viruses. An *ori-Lyt* plasmid (pOri-A) was introduced into 293T cells harboring BAC36 or K8-null mutant genomes, respectively. The lytic replication was induced by cotransfecting cells with an RTA expression vector. The replication of *ori-Lyt* plasmid in cells was detected by using DpnI digestion as described above. Findings from this experiment showed similar replication levels of the *ori-Lyt* plasmid in both cells carrying wild-type BAC36 or the K8-null mutants (Fig. 5A). K8 was also introduced into cells carrying K8-null mutant viruses to see whether K8 expression has any effect on viral lytic DNA replication. In this experiment, though it was initially presumed that overexpression of K8 increased the DNA replication, subsequent normalization of the replicated bands with input DNA showed no significant effect of K8 expression on the DNA replication (Fig. 5B). Taken together, our data suggest that K8 is not absolutely required for KSHV lytic DNA replication.

K8-null mutant viruses replicate in B lymphocytes. K8-null mutant viruses replicating in 293T cells was an unexpected observation. We were thus tempted to investigate whether K8-independent viral lytic DNA replication also occurs in

FIG. 4. Lytic replication of BAC-cloned wild-type and K8-null KSHV genomic DNAs. BAC36, BAC-ΔK8, and BAC-stopK8 DNAs were introduced into 293T cells by transfection, along with an RTA expression vector (pCR3.1-ORF50) or K8 expression vector (pCR3.1-K8α) as indicated. KSHV lytic replication was induced by expression of RTA. Total DNAs were isolated from the transfected cells and used in the replication assay. Replicated bacmid DNAs were distinguished from input DNAs by DpnI digestion and detected by Southern blotting with ^{32}P -labeled DNA fragment of the LANA coding sequence.

FIG. 5. K8-null recombinant viruses support replication of KSHV *ori-Lyt*-containing plasmid in the lytic phase. 293T cells carrying wild-type or K8-null recombinant KSHV genome were transfected with *ori-Lyt* plasmids (pOri-A) plus others as indicated (pCR3.1-ORF50 and pCR3.1-K8α). KSHV lytic replication was induced by expression of ORF50 (RTA). Total DNAs were isolated from the transfected cells and used in the replication assay. (A) Replicated DNAs were distinguished from input DNAs by DpnI digestion and detected by Southern blotting with ³²P-labeled pBluescript plasmid. The replicated plasmid DNAs were quantitated by PhosphorImager scanning and normalized with their total plasmid DNAs (without DpnI digestion). (B) The replication rate of pOri-A DNA in each cell line was calculated by comparing the intensity of the replicated (Rep'd) DNA band with the input DNA bands.

other types of cells. To address this issue, we introduced BAC-cloned wild-type (BAC36 and BAC-revK8) and K8-null (BAC-ΔK8 and BAC-stopK8) viral genomes into the EBV-negative B lymphoblastoid BJAB cell line. Since the rate of transfection of BAC-cloned viral genome to B lymphocytes is very low, the transfected cells were under hygromycin selection until the majority of cells became green (spanning a time period of 4 to 6 weeks). The cells were induced by TPA and butyrate and

subjected to Gardella gel analysis. As showed in Fig. 3B, replicated form of viral DNA was detected in both wild-type and K8-null viruses.

The intracellular viral genomic DNA contents in BAC-cloned KSHV DNA-transfected BJAB cells prior to and after TPA-butyrate induction were determined by analyzing intracellular DNAs using real-time PCR. Our findings indicated 6- to 7-fold increases in intracellular viral DNA in both wild-type

FIG. 6. Lytic DNA replication and extracellular progeny virion production of K8-null recombinant viruses in BJAB lymphocytes. BAC-cloned wild-type (BAC36 and BAC-revK8) and K8-null (BAC-ΔK8 and BAC-stopK8) viral genomes were introduced into BJAB cells by electroporation. Transfected cells were enriched through a hygromycin selection. Selected cells were treated with TPA and sodium butyrate to induce KSHV lytic replication. (A) Two days postinduction, cells were collected and total intracellular DNA was extracted with the Qiagen DNeasy kit. Intracellular viral DNAs were measured by a real-time PCR with primers directed to the ORF73 gene from a 100-ng input DNA sample. The viral genome copies were normalized to 20,000 copies of GAPDH. (B) Five days postinduction, extracellular KSHV virions were harvested and concentrated 100-fold. Virus stocks were treated with Turbo DNase I, and viral genomic DNA was extracted. KSHV genomic DNA concentration was estimated by a real-time PCR, along with external standards of known concentrations of the viral DNA with primers against the ORF73 gene. Virus titers were presented as viral DNA copy numbers per milliliter of the supernatant.

and K8-null mutant viruses upon TPA-butyrate induction for 48 h. No significant difference was observed between wild-type and K8-null viruses in this regard (Fig. 6A). These findings indicate the nonrequirement of K8 toward KSHV lytic DNA replication even in B lymphocytes.

Effect of K8 deficiency on progeny virion production. Next, we investigated whether K8 deficiency has any effect on production of progeny virions. The pooled hygromycin-resistant and GFP-positive 293T/BJAB cells were induced with TPA/n-butyrate for 5 days. Viral particles were collected from the

culture media and passed through 0.45-μm-pore-size filters. Because a typical plaque assay is not available for KSHV, we estimated the amounts of virus by quantifying encapsidated viral DNA in preparations (35). We first treated the concentrated viruses with Turbo DNase I (Ambion) at 37°C for 1 h to remove any contaminating DNA outside viral particles. The viral DNAs were extracted and analyzed with real-time PCR. From a standard curve with known amounts of BAC36 DNA, the copy numbers of viral genomes in induced media were estimated. Extracellular virion titers obtained from both BJAB

FIG. 7. Lytic DNA replication and extracellular progeny virion production of K8-null recombinant viruses in 293T cells. BAC-cloned wild-type (BAC36) and K8-null (BAC-ΔK8 and BAC-stopK8) viral genomes were introduced into 293T cells by transfection. The transfected cells were enriched through a hygromycin selection. The pooled hygromycin-resistant cells were induced with TPA and sodium butyrate to induce KSHV lytic replication. (A) At 2 days postinduction, cells were collected, and total intracellular DNA was extracted with a Qiagen DNeasy kit. Intracellular viral DNA was measured by a real-time PCR with primers directed to the ORF73 gene from a 100-ng input DNA sample. The viral genome copies were normalized to 20,000 copies of GAPDH. The fold induction of each BAC clone upon TPA/*n*-butyrate are indicated above the bars. (B) At 5 days postinduction, extracellular KSHV virions in the media were harvested and concentrated 100-fold. Virus stocks (200 μl) were treated with Turbo DNase I for 1 h at 37°C, and viral DNAs were extracted. Viral DNAs were analyzed by a real-time PCR assay using primers to LANA. A serial dilution of a known amount of BAC36 DNA was used to construct a standard curve. Copy numbers were normalized and are expressed as the copy number per milliliter of supernatant.

and 293T cells are shown in Fig. 6B and 7B, respectively. 293T cells with K8-null viruses had lower virion titers than cells with wild-type virus (Fig. 7B). Intracellular KSHV genomic DNA in these cells was also analyzed. The cells carrying wild-type and mutant viruses were treated with TPA-butyrate for 3 days. DNA was isolated from the cells and the intracellular KSHV genomic DNA was analyzed using real-time PCR. As shown in Fig. 7A, the KSHV genome copy numbers in K8-null viruses was lower than that of wild-type viruses both prior to and after TPA-butyrate induction. There was, however, no statistical difference in the rates of lytic DNA replication between wild-type and mutant viruses (Fig. 7B). These findings suggested that the lower intracellular KSHV genome copy numbers ob-

tained in TPA-induced 293T-K8-null cells may not be due to reduced rate of viral lytic DNA replication, but rather due to lowered latent KSHV episomal DNA copies in these cells. However, the association of K8 deficiency and lower latent viral DNA content was not obvious in BJAB cells harboring K8-null KSHV mutants. A possible explanation for this observation could be due to the transfected BJAB cells being subjected to a heavy drug selection over a prolonged period of time, presumably resulting in enrichment of viral genomic DNA in non-TPA-induced BJAB cells.

To rule out the possibility that the lowered intracellular KSHV DNA content and the reduced virion production observed with the K8-null viruses was not caused by K8 deficiency

FIG. 8. Kinetics of lytic DNA replication and extracellular progeny virion production associated with different clones of K8-null recombinant viruses in 293T cells. Three independently derived K8 deletion (BAC-ΔK8-1, BAC-ΔK8-2, and BAC-ΔK8-3) and three K8-stop-codon (BAC-stopK8-1, BAC-stopK8-2, and BAC-stopK8-3) mutants were introduced into 293T cells. Each transfected cell line was selected with hygromycin and then induced with TPA and butyrate to induce KSHV lytic replication. (A) At different time points postinduction (0, 24, 48, and 72 h), cells were collected, and total intracellular DNA was extracted with a Qiagen DNeasy kit. Intracellular viral DNAs were measured by a real-time PCR with primers directed to the ORF73 gene from 100 ng of input DNA. The viral genome copies were normalized to 20,000 copies of GAPDH. (B) At different time points of postinduction (0, 24, 48, 72, and 120 h), extracellular KSHV virions in the media were harvested and concentrated 100-fold. Virus stocks were treated with Turbo DNase I for 1 h at 37°C, and viral DNAs were extracted. Viral DNAs were analyzed by a real-time PCR assay using primers to ORF73. A serial dilution of a known amount of BAC36 DNA was used to construct a standard curve. Copy numbers were normalized and are expressed as copy number per milliliter of supernatant.

but instead was due to the variation of individual viral clones, we repeated the recombineering procedure and independently isolated two more BAC-stopK8 clones. We introduced the three individual clones of BAC-stopK8 (BAC-stopK8-1, -2, and -3) into 293T cells. In addition, two more BAC-ΔK8 mutants (BAC-ΔK8-2 and BAC-ΔK8-3) generated as described above, together with BAC-ΔK8-1 (from which all of the BAC-stopK8 and BAC-revK8 were derived), were also included in the assay. Transfected 293T cells were induced with TPA and

sodium butyrate, and the intracellular KSHV genomic DNAs were isolated from these cells at 0, 24, 48, and 72 h postinduction. The extracellular virion DNAs were purified from virion particles released to the media at 24, 48, 72, and 120 h postinduction. Both intracellular and virion DNAs were analyzed by a quantitative real-time PCR as described above. Prior to TPA-butyrate induction, the levels of intracellular KSHV genomic DNA in all three BAC-ΔK8 and BAC-stopK8 clones was only 30 to 40% of the levels obtained with wild-type viruses

FIG. 9. Infectivity of reconstituted BAC36 and K8-null viruses. 293T, human foreskin fibroblasts (HFF), and human microvascular endothelial cells (HMVEC) plated in 24-well plates were incubated with concentrated virus (50 genome copies per cell) and spun at 2,500 rpm for 1 h at room temperature. The inocula were then removed and replaced with fresh medium plus 5% FBS. The following day, the media were replaced with fresh medium containing 1% FBS. GFP expression was examined under a fluorescence microscope at 2 days postinfection.

(BAC36 and BAC-revK8). After induction, though the intracellular viral DNA contents increased 4- to 6-fold, the overall viral DNA contents in the K8-null mutant-carrying cells was still lower than that obtained with the wild-type virus-carrying cells (Fig. 8A). In addition, all of the three BAC-ΔK8 and BAC-stopK8 mutant viruses released lower virion particles into the medium compared to the wild-type viruses (Fig. 8B).

Infectivity of K8-null recombinant virus and the early event after infection. To determine the infectivity of the K8-null virus, BAC-ΔK8 and BAC-stopK8, along with the wild-type BAC36 viral particles, were concentrated from the culture media of TPA-butyrate-treated 293T cells carrying the corresponding viral genomes and used to infect target cells at 50 genomes per cell. For the viral infectivity studies, three target cells were chosen: 293T, HMVEC-d, and HFF cells. The infectivity of mutant and wild-type viruses was found to be similar, as revealed by the appearance of green cells after primary infection of all of the target cells (Fig. 9). This result was not surprising given that virion particles of both wild-type and K8-null mutants would be identical since K8 is not incorporated into virion particles (2, 31).

Subsequently, we determined the kinetics of viral genome entry and replication in the above three target cell types. Total DNA was extracted from infected cells at different time points postinfection up to a time period of 72 h, and the KSHV genome copy numbers were determined by a real-time PCR as described above. Internalized wild-type viral copy numbers increased during the first 2 h (293T and HFF cells) to 4 h (HMVEC-d cells) of infection and reached a plateau. The plateau remained for a few hours in 293T and HFF cells and for at least 48 h in HMVEC-d cells before the viral copy numbers started to plunge (Fig. 10). Compared to wild-type virus, both BAC-ΔK8- and BAC-stopK8-infected cells exhibited much lower genome copy numbers in all cell types (Fig. 10). These unexpected data were both reproducible and con-

sistent, with only minor variations between experiments and batches of viruses.

To confirm the association of K8 deficiency with the lower KSHV genomic copy numbers in K8-null mutant-infected cells, 293T cells were transfected with K8 expression vector (pCR3.1-K8α) and then infected with K8-null mutant viruses. KSHV genomic DNA content in the infected and transfected cells was determined by a real-time PCR. Ectopic expression of K8 protein compensated the deficiency of K8 in both BAC-ΔK8-infected (Fig. 11A) and BAC-stopK8-infected cells (Fig. 11B), yielding a higher intracellular viral genomic DNA content.

Lytic mode of viral DNA replication in the early stage of *de novo* KSHV infection. The kinetic analysis for the viral DNA replication in the early stage of viral infection showed a possible role of K8 in this process. However, details on the DNA replication at this stage has largely remained elusive, especially pertaining to the mode of viral DNA replication with respect to it being latent or lytic. To approach this issue, we treated KSHV-infected cells with phosphonoacetic acid (PAA), which inhibits herpesviral replication by inactivating the viral DNA polymerase. 293T cells were infected with purified KSHV virions and infected cells cultured in the presence of PAA. The viral genomic DNA content in wild-type KSHV (BAC-36)-infected cells was dramatically decreased to levels close to that witnessed in the K8-deficiency virus (BAC-stopK8)-infected cells (Fig. 12). This observation raises the possibility that there is a viral DNA polymerase-dependent DNA replication occurring in the early stage of *de novo* infection with K8, possibly playing a critical role in this initial viral DNA replication.

The feasibility of the hypothesis described above arises from the following observations. (i) It was reported that a limited subset of KSHV lytic genes are expressed during very early stage of *de novo* infection inclusive of ORF9 encoding KSHV DNA polymerase, expressed 2 and 8 h postinfection of HMVEC-d and HFF cells, respectively (13), suggesting the possible participation of the viral DNA polymerase in viral DNA replication processes immediately after infection. (ii) K8 protein was detected 2 and 8 h postinfection of HMVEC-d and HFF cells, respectively, with decreases noticed at later time points, suggesting a role of K8 in viral DNA replication at that stage (13). (iii) It is known that *de novo* infection by gamma-herpesviruses, including EBV and KSHV, in general leads to a default latency. Although the *de novo* virus protein synthesis is tightly blocked following a primary infection, an abortive lytic-phase viral replication is known to occur, culminating in increases in viral genome copy numbers to approximately 50 to 100 copies per cell. Taken together, our results suggest a possible role of K8 in abortive lytic DNA replication occurring in the early stage of *de novo* KSHV infection.

DISCUSSION

The function of the ORF K8-encoded bZip protein has remained elusive since its identification. Based on the fact that the EBV analogue of K8, namely, Zta or ZEBRA, is a transcriptional activator that activates the viral lytic gene expression cascade and initiates the switch of EBV from latency to lytic life cycle (21), K8 bZip protein was also speculated to be a transcription factor similar to its EBV counterpart. However, despite extensive studies in several laboratories, no transcrip-

FIG. 10. Kinetics of entry and replication of wild-type and K8-null viruses into HMVEC-d (A), 293T (B), and HFF cells (C). HMVEC-d, 293T, and HFF cells were infected with either the concentrated wild-type or the K8-null viruses at an MOI of 50 KSHV DNA copies per cell for 1 h. The inocula were then removed and replaced by fresh media. At different time points postinfection, total DNA was isolated from infected cells, and viral genomic DNAs were quantified by real-time PCR with primers specific for ORF73. Viral DNA copy numbers were calculated from a standard graph generated using known concentrations of BAC36 DNA.

tional activity was found to be associated with K8. Instead, it was reported that K8 is a transcription repressor downregulating the transcription of a subset of viral and cellular genes (10, 16). It has been shown that K-bZIP/K8 is in fact a sumoylated protein with the associated sumoylation activity contributing to the protein's transcriptional repression activity (11). K8 was

found to bind to the KSHV genome at the origins for viral DNA lytic replication (*ori-Lyt*), and this binding was shown to be essential for viral lytic DNA replication. The direct evidence as to the pivotal role of K8 toward KSHV lytic DNA replication came from findings of a transient-transfection assay, which showed that KSHV *ori-Lyt*-dependent DNA replication re-

FIG. 11. Reversal of K8 deficiency-associated lowered intracellular viral DNA by complementation of K8-null mutant viruses with the epigenetic expression of K8. 293T cells were transfected with K8 expression vector (pCR3.1-K8 α). At 24 h posttransfection, transfected and untransfected cells were infected with wild-type (BAC36) and K8-null (BAC- Δ K8 and BAC-stopK8) viruses. At different time points postinfection, total DNA was isolated from infected cells, and viral genomic DNA was quantified by real-time PCR with primers specific for ORF73. Viral DNA copy numbers were calculated from a standard graph generated using known concentrations of BAC36 DNA.

quires six core replication proteins with the additional presence of K8 and RTA, and omission of K8 in the transient-transfection assay abolished the viral DNA replication (1). However, these observations were made under the circumstances involving over expression of K8 in a nonviral context. To investigate the genuine functions of K8 in viral life cycle, we generated K8-null viruses that allowed us to conveniently and reliably investigate the functional roles of K8 in the context of the actual viral life cycle. Using these viruses, we demonstrated the dispensability of K8 toward viral lytic DNA replication which was rather surprising. More interestingly, our study indicated a possible role of K8 in the control of the viral latent episomal genomic number in latently infected cells, an important observation worth pursuing.

Recently, Kato-Noah et al. (12), following generation of a K8 deletion mutant virus, have illustrated (i) the failure of this mutant to replicate and produce infectious virions after TPA/*n*-butyrate induction and (ii) the dispensability of K8 toward DNA expression subsequent to RTA overexpression. These observations were discrepant to our study findings, wherein, using the K8-null recombinant viruses, we documented the nonrequirement of K8 toward lytic viral DNA replication and virion production even in the absence of RTA overexpression. In our laboratory we have frequently noticed that BAC-cloned KSHV genomes, both wild type and mutants, are unstable in mammalian cells and are potently induced by TPA and butyrate only in freshly transfected cells. Long-term (typically involving time periods spanning more than 2 months) selection

FIG. 12. Effect of phosphonoacetic acid (PAA) on initial DNA replication of wild type (BAC36) and K8-null (BAC-stopK8) following *de novo* infection. 293T cells were infected with concentrated wild-type and K8-null viruses at an MOI of 50 KSHV DNA copies per cell for 1 h. The inocula were then removed and cells were incubated in fresh media with or without 0.4 mM PAA. At different time points postinfection, total DNA was isolated from infected cells and viral genomic DNAs were quantified by real-time PCR with primers specific for ORF73. Viral DNA copy numbers were calculated from a standard graph generated using known concentrations of BAC36 DNA.

and culture of the BAC transfected cells invariably resulted in loss of ability of the cells to be efficiently induced by TPA and butyrate. We thus opine that such technical difficulties associated with the induction of BAC-cloned KSHV genome into the lytic life could have contributed to the discrepancies noticed between the two studies.

The dispensability of K8 toward viral lytic DNA replication in both 293T and B lymphocytes was rather unexpected, even though undeniable evidence shows K8 as an integral component of viral DNA replication complexes and is itself localized to the viral replication compartment (1, 25–28). Although it is tempting to speculate that K8 may only play an accessory role in KSHV lytic DNA replication or is merely a bystander in the viral replication complex, we are more leaning toward the possibility that K8 could still have a critical role in viral replication, though some cellular proteins with similar structural or functional properties (such as C/EBP α) could substitute for it, thus compensating for its deficiency. It may thus be definitely worthwhile to test whether K8 becomes essential for viral DNA replication under the condition where C/EBP α expression is knocked down.

The most surprising and exciting observation with our K8-null mutant virus system was the finding that the K8-null mutant viruses exhibited lower levels of viral DNA replication in the early stage of *de novo* infection and also maintained lower KSHV genome numbers during latency. A unique feature of gammaherpesviruses, including EBV and KSHV, is that primary infection leads to the latency by default where the synthesis and release of progeny virus does not occur. Although the *de novo* virus synthesis is tightly blocked, an abortive lytic phase takes place, in which a limited number of lytic viral genes are expressed and viral genome copy numbers increase to approximately 50 to 100 copies per cell. Our result raises a possibility that K8 may be involved in the DNA replication in the abortive lytic phase. K8 and RTA have been shown to be

transiently expressed in the very early hours after viral infection, together with a limited subset of lytic cycle genes, including the viral DNA polymerase and two more DNA replication related proteins (13). The increases in the intracellular viral genomic DNA content following *de novo* infection may at least partially represent the abortive lytic viral replication. Taking all of these into consideration, we hypothesize that (i) there is a viral DNA polymerase-dependent viral DNA replication immediately following viral entry into host cells accompanied with transcription of a limited number of viral lytic cycle genes, (ii) this initial lytic DNA replication is not successful enough to proceed to a full lytic cycle of viral replication but is competent enough to increase the viral genome number to 50 to 100 copies per cell, and (iii) K8 appears to play a role in the abortive lytic DNA replication.

Currently, almost nothing is known about the DNA replication in the abortive lytic phase in gammaherpesviruses. Information is lacking on whether *ori-Lyt*, *ori-P*, or other origins are used to initiate viral DNA replication in this phase. Given the possibility of *ori-Lyt* being involved in the DNA replication at this stage, it is tempting to speculate that K8 may recruit viral DNA polymerase and some cellular DNA replication enzymes/factors to support viral DNA replication in a situation where many viral components for lytic DNA replication are not expressed. Thus, the absence of K8 could potentially impair this recruitment, resulting in a defective abortive lytic phase. In addition, it was recently demonstrated that LANA represses origin-dependent lytic DNA replication with this repression being relieved by the presence of K8 protein (22). Thus, arises a second hypothetical model concerning the role of K8 in the abortive lytic DNA replication, wherein K8 could potentially counteract LANA-mediated repression of lytic mode of viral DNA replication in the early stage of infection. The second model appears to be supported by our recent observation that treatment of either the BAC36 or the BAC-stopK8 recombi-

nant virus-infected cells, with wortmannin, a phosphatidylinositol 3-kinase (PI3K) inhibitor known to block the PI3K signal transduction, resulting in inhibition of LANA expression, completely relieved the repression of abortive lytic DNA replication in the absence of K8 (unpublished data). Thus, in essence, the present study has opened up avenues toward further elucidating the functional role of K8 in the initial DNA replication following primary KSHV infection.

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