The Journal of Microbiology (2012) Vol. 50, No. 2, pp. 285–292
DOI 10.1007/s12275-012-1495-9
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KSHV Infection of B-Cell Lymphoma Using a Modified KSHV BAC36 and Coculturing System[§]

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(Received October 4, 2011 / Accepted November 15, 2011)

Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of two B cell lymphoproliferative diseases, namely primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). KSHV infection of B cell lymphoma *in vitro* has been a long-standing battle in advancing human KSHV biology. In this study, a modified form of KSHV BAC36 named BAC36A significantly increased the fidelity of gene-targeted site-directed mutagenesis in the KSHV genome. This modification eliminates tedious screening steps required to obtain mutant clones when a KSHV BAC36 reverse genetic system is used. Coculturing B-cell lymphoma BJAB cells with KSHV BAC36A stably transfected 293T cells enabled us to infect BJAB cells with a KSHV virion derived from the KSHV BAC36A. The coculture system produced substantial amounts of KSHV infection to BJAB, meaning that KSHV virions were released from 293T cells and then infected neighboring BJAB cells. Owing to our success with the KSHV BAC36A and coculture system, we propose a new genetic system for the study of KSHV gene expression and regulation in B-cell lymphoma.

Keywords: KSHV, B cell lymphoma, coculture, KSHV BAC36

Introduction

Human herpesvirus 8 called Kaposi's sarcoma-associated herpesvirus (KSHV) has been identified in Kaposi's sarcoma (KS) tumors, primary effusion lymphoma (PEL) and multicentric Castleman's disease (Ganem, 2006). Due to the significant sequence homology to gammaherpesviruses, it has become the latest addition in the gammaherpesvirus family, which already includes Epstein-Barr virus (EBV), herpesvirus saimiri and murine gammaherpesvirus 68 (MHV-68) (Mesri *et al.*, 2010). B cells are the major target for other KSHV-like gammaherpesviruses, and B cell lines such as BCBL-1, BC-1, and BC-3 have been widely used in KSHV replication studies (Mesri *et al.*, 1996; Renne *et al.*, 1996). Despite the prevalence of KSHV infection in B cells *in vivo*, KSHV B cell infection studies have been established in only a few *in vitro* infection models.

KSHV has a characteristic latent infection in lymphoid cells where KSHV expresses a limited number of latent genes (Renne *et al.*, 1998; Sadler *et al.*, 1999). Representative latent genes encode v-cyclin (ORF72), latency-associated nuclear antigen (LANA, ORF73), v-FLIP (ORF71), kaposin (K12), miRNAs, and K10.1 (LANA-2). KSHV switches to lytic replication cycles when the viral latency is disrupted by external or internal factors. Similar to latent gene expression, the KSHV lytic genes turn on in a temporal and sequential order such as immediate-early, early, and late intervals (Deng *et al.*, 2007; Greene *et al.*, 2007). Replication and Transcription Activator (RTA), a key lytic reactivation switch molecule, is a potent immediate-early transcription activator for most KSHV lytic genes (Chen *et al.*, 2009). Owing to this distinct replication cycle, it was very tempting to study KSHV genetics using a reverse genetic system, which, unfortunately, is not available in lymphocytic cells.

KSHV genetics greatly advanced thanks to a KSHV genome expressed in a bacterial artificial chromosome (KSHV-BAC36) (Zhou *et al.*, 2002; Rezaee *et al.*, 2006; Yakushko *et al.*, 2011). A BAC cassette was inserted between ORF18 and ORF19 of the KSHV genome derived from BCBL-1 cells (position 33195 of the reference sequence; GenBank accession no. NC009333), which generates the KSHV-BAC36 (Rezaee *et al.*, 2006). The KSHV-BAC36 is, so far, the only genetic system that allows us to introduce point mutations into the KSHV genome. BAC36 can be transfected and maintained in 293T cells for at least 6 weeks. The 293T cells are also permissive to infection by the KSHV virion derived from BAC36. Due to these features, several genetic studies were conducted using the BAC36. However, unlike the 293T cells' permissiveness to KSHV infection, most established B cell lines are resistant to either stable BAC36 transfection or KSHV virion infection. In addition, the KSHV BAC36 has been found to be very unstable to homologous recombination, which resulted in low yields in gene-targeted, site-directed mutagenesis. Thus, it was necessary to stabilize the KSHV BAC36 and thereby increase the fidelity of gene-targeted, site-directed mutagenesis.

To address these concerns regarding BAC stability and easy access to reverse genetics, we introduced new modifications into KSHV BAC36. The ampicillin resistance gene was inserted at ORF69 and K12, which prevented the KSHV-BAC36 from deforming during homologous recombination. This actually increased the BAC36 stability enough to significantly enhance (approximately 500 times) the fidelity of

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[§]Supplemental material for this article may be found at http://www.springer.com/content/120956

gene-targeted, site-directed mutagenesis. We also developed a KSHV B-cell infection model by co-culturing KSHV negative B-cell lymphoma BJAB and KSHV BAC36 positive 293T cells. BJAB cells infected with the KSHV virion could be maintained, albeit for limited periods. These results demonstrate that the modified KSHV BAC36 and B-cell infection system will be useful for studying KSHV biology in B cell lymphomas.

Materials and Methods

Cells and media

The KSHV-positive primary effusion lymphoma cell line BCBL1 and the KSHV-negative B-lymphoma cell line BJAB were cultured at 37°C and 5% CO_2 in RPMI medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics/antimycotics (Anti-Anti, Promega, 1%). The 293T human embryonic kidney cell line (ATCC) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% Anti-Anti. The 293T-derived cell lines with KSHV BAC transfection were all cultured the same as 293T cells, except for the addition of 200 μg/ml hygromycin B for the selection of the wild-type (WT) and recombinant viral genomes.

Modification of BAC36

KSHV BAC36 was modified to facilitate the efficiency of site-specific mutation into the DNA region of interest of the viral genome. The Amp resistance gene (Amp^r gene) was amplified from the pGEM-T vector. The resultant 908 bp Amp^r gene was introduced using a Counter-Selection BAC Modification Kit (Gene Bridgers) at KSHV 117647, where the polypyrimidine tract binding protein (PTB) binding sites (AATAA) for ORF69 and ORFK12 are located 293 bp upstream and 236 bp downstream, respectively. The insertion sequence was confirmed by sequencing, and its orientation was found to be in parallel to ORF69. The resultant KSHV BAC36 with Amp insertion was named as KSHV BAC36-Amp (BAC36A). A site-specific mutation was introduced into the identified CCCTC-binding factor (CTCF)/Cohesin binding sites (KSHV genome 127376-127529) as previously described (Stedman *et al*., 2008). Homologous recombination using the Counter-Selection BAC Modification Kit was conducted twice to generate KSHV BAC36A carrying point mutations in the CTCF/Cohesin binding sites. The resultant mutant BAC was confirmed to have a targeted point mutation by sequencing and then named as CC-mt1. Detailed procedures for generating CC-mt1 were described in our previous study (Kang *et al*., 2011).

Confirmation of KSHV BACs

A bacterial clone (*E. coli*, ElectroMAX™ DH10B™ cells) was inoculated in 4 ml LB medium (antibiotics plus) and cultured overnight with vigorous shaking at 37°C. Grown *E. coli* (DH10B) were harvested by centrifugation at 11,000×g for 2 min. After aspirating the culture supernatant, the pellet was resuspended completely in 200 μl resuspension buffer (50 mM Tris-HCl; pH 8.0, 10 mM EDTA, 20 μg RNase A), then 200 μl lysis buffer (200 mM NaOH, 1% SDS) were added and mixed gently at room temperature. Neutralization buffer (240 μl: 3 M potassium acetate, pH 5.5) was added and mixed gently for 5 min at room temperature. Bacterial debris was removed by centrifugation at 11,000×g for 5 min and the supernatant was transferred to a fresh 1.5-ml microcentrifuge tube. 600 μl of P.C.I. (phenol: chloroform: isoamyl alcohol = 25:24:1) were added and mixed well for 2 min. Proteins were removed by centrifugation at 11,000×g for 5 min and then transferred to a fresh 1.5-ml microcentrifuge tube. Following addition of 2 μl of glycogen (20 μg/μl), 600 μl isopropanol were added to the BAC DNA solution and mixed thoroughly for 10 min at room temperature. The BAC DNA pellet was collected by centrifugation at 11,000×g for 10 min, followed by washing of the DNA pellet with 750 μl of 70% ethanol. After removing all traces of ethanol, the BAC DNA pellet was dissolved in 50 μl of sterile distilled water without air-drying. SacI restriction enzyme digestion (NEB, 40 U per 50 μl reaction volume) was set up to digest BAC DNA overnight. *Sac*I restriction sites on the KSHV genome are 102899, 103063, 103789, 107940, 115051, 115707, 115714, 122592, and 127394. Either a point mutation or Lox P substitution mutation eliminated the SacI restriction site located at KSHV genome 127349, thus removing a distinct 4.8-kb DNA fragment when the BACs carrying mutations in the CTCF/Cohesin binding sites were digested by *Sac*I restriction enzyme.

Transfection

Effecten (QIAGEN, USA) was used to transfect 293T cells with KSHV BACs, whose CTCF- binding sites in major latency control regions were removed by site-directed mutagenesis. The transfected 293T cells were selected under hygromycin B (1,000× dilution) for 3 weeks by replacing complete DMEM medium every 2 days, and resultant the GFP^+ 293T cells were named as 293T-KSHV cells.

Intracellular episome copy numbers

A pcDNA3-based vector, overexpressing KSHV RTA under control of the CMV promoter, was transfected into 293T cells and RTA-mediated trans-activation of KSHV reactivation in 293T-KSHV cells was assessed 24, 48, and 72 h post-transfection. Using methods previously described (Kang *et al*., 2011), intracellular KSHV episome copy numbers were determined relative to cellular GAPDH.

BJAB infection by coculturing

Effecten (QIAGEN) was used to transfect 293T cells with KSHV BAC36-AmP (BAC36A) or the BAC36A carrying a point mutation in the CTCF/Cohesin binding sites (CC-mt1), and then the cells were selected with hygromycin (20 μg/ml) for approximately two weeks. A sample of 2×10^6 cells of selected 293T-BAC36 or CC-mt1 was transfected with KSHV RTA overpexression constructs (pcDNA3, Invitrogen, USA) and further incubated at 37°C for 48 h. To remove cell debris, old culture medium (DMEM) was filtered through a 0.22 μm Millipore filter and the RTA transfected 293T-KSHV cells were rinsed twice with PBS.

The old DMEM medium was added back to the RTA-transfected 293T-KSHV cells where 4×10^6 BJAB cells were overlayed for coculturing. The 293T-BJAB cells in the co-culture were incubated for a further 48 h, and BJAB cells infected by KSHV virions were then sorted using FACs Aria III (BD Science). Sorted BJAB cells were GFP^+ and $CD45^+$, and maintained with hygromycin (5 μg/ml) until 10 days post-sorting for analysis.

Staining with PE-Cy™ 7 mouse anti-human CD45

Suspended cells were recovered from the coculture of 293T-KSHV and BJAB cells after gentle up and down shaking, and then pelleted. The cells were washed twice in cold staining buffer, pelleted by centrifugation (1,200 rpm, 5 min), resuspended with cold staining buffer to a final concentration of 2×10^7 cells/ml, and 100 μl aliquots of the cell suspension (2×10^6 cells) were distributed to tubes (12× 75 mm polypropylene round-bottom test tube). PE-CyTM 7 Mouse anti-Human CD45 (50 μl) was added to cells followed by a 20 min incubation on ice with protection from light for CD45 surface staining. The resultant CD45-stained cells were twice washed with 1 ml of stain buffer, pelleted at 1,200 rpm for 5 min, and resuspended in 0.5 ml of stain buffer for flow cytometry sorting and analysis.

RT-qPCR

Reverse transcriptase quantitative PCR (RT-qPCR) was used to determine transcription profiles of KSHV gene expression in BJAB. The RT-qPCR protocol was described previously (Kang and Liberman, 2009).

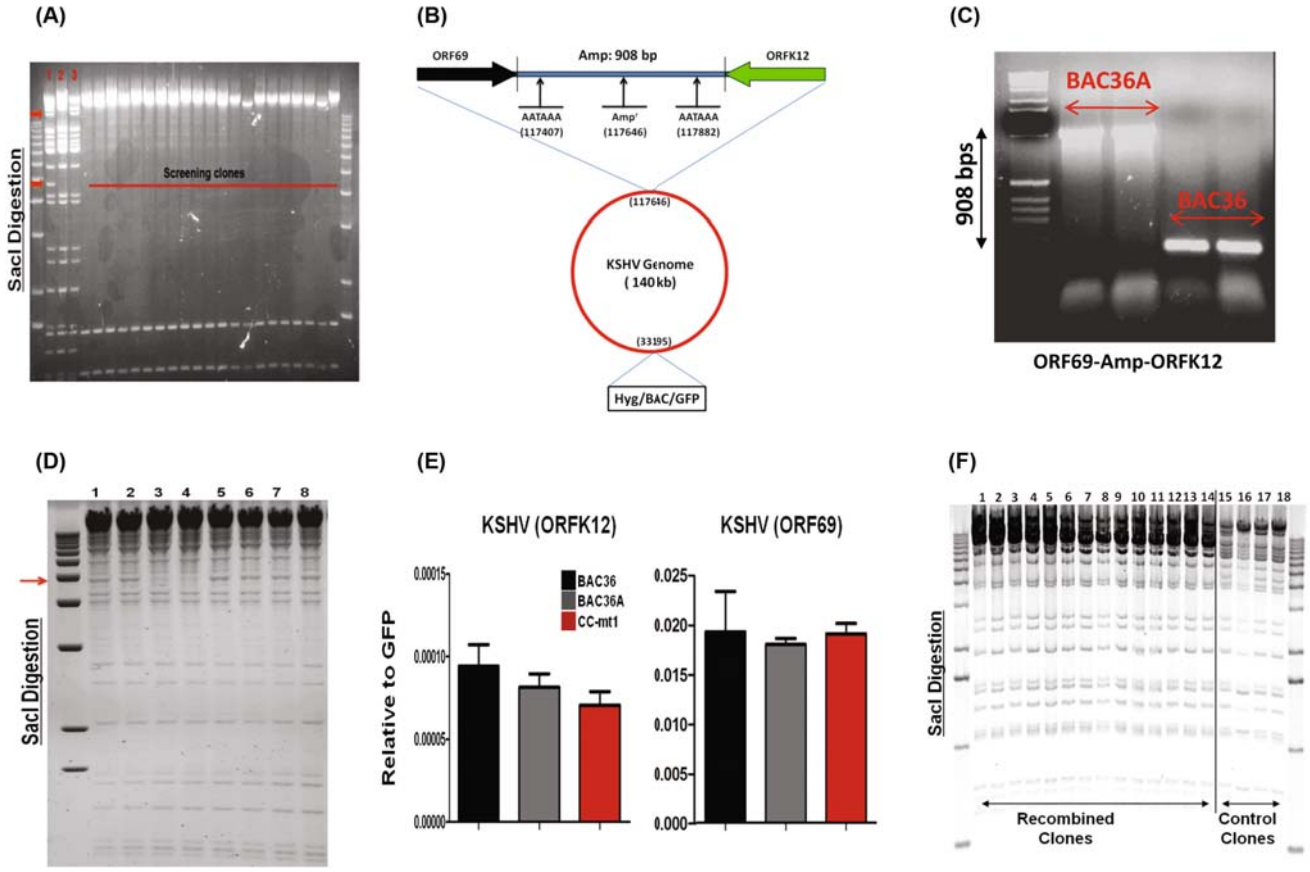

Fig. 1. Manipulations of KSHV BAC36. (A) Deformation of KSHV BAC36 resulting from two distinct Red/ET recombinations aimed at inserting a non-selectable marker gene into BAC36: Lane 1 was loaded with parental KSHV BAC36, lanes 2-3 with LoxP CTCF Mutant BAC N1108, and the of the remaining lanes with screened clones (21 clones). (B) Schematic diagram showing insertion of the Amp^r gene into KSHV BAC36: The Ampr gene was inserted at the opposite side of where the Chl^r gene was located on KSHV BAC36. (C) PCR-based confirmation of the Ampr gene insertion in KSHV BAC36: Insertion of the Ampr gene was confirmed by conventional PCR. Using primers designed to flank the inserted Amp^r gene, the PCR assay determined the presence of the 906-bp Amp^r gene in the KSHV BAC36A genome. (D) Verification of KSHV genome integrity after Amp^r gene insertion by *Sac*I genomic digestion: The *Sac*I restriction site at KSHV genome bp 127394 was removed by LoxP insertion at the CTCF/Cohesin binding sites (KSHV genome bp 127376-127529). Lanes 1-2 were loaded with parental KSHV BAC36, lanes 3–4 with LoxP CTCF Mutant BAC N1108, and lanes 5-8 with KSHV BAC36A. (E) Effect of the Amp^r gene insertion on the production of ORF69 and ORFK12 transcripts: Transcription of KSHV ORF69 and ORFK12 was tested in 293T-KSHV cells to determine, using RT-qPCR, if Amp^r gene insertion caused defects in ORF69 and ORFK12 expression. (F) Confirmation of the CTCF mutation in KSHV BAC36A by *Sac*I digestion assay: KSHV BAC36A carrying the CTCF mutation (CC-mt1) was identified by *Sac*I digestion of the BAC genome. Lanes 1-14 were loaded with site-directed CTCF mutant BAC CC-mt1 (clone #1-14), lane 15 with parental KSHV BAC36, lane 16 with LoxP CTCF Mutant BAC, N1108, and lanes 17-18 with KSHV BAC36-Amp (BAC36A).

Results

BAC36A showed significantly higher efficiency in homologously recombined KSHV BACs than KSHV BAC36

The Counter-Selection BAC Modification Kit was used to generate the KSHV BAC36 that carries point mutations in the CTCF/Cohesin binding sites (CC-mt1). Unexpectedly, BAC36 was frequently deformed by spontaneous additional recombination when Red/ET recombination replaced the rpsL-neo counter selection/selection cassette with the 1-kb DNA fragment carrying a site-directed mutation in the CTCF/Cohesin binding sites (Fig. 1A). Although we screened more than 500 DH10B clones that survived antibiotic selection, no clone harboring the targeted point mutations in KSHV BAC36 was found. Moreover, we observed KSHV BAC36 instability when BAC36 was transformed into the modified DH10B strain SW102. Several BAC modifications using a galK positive/counter selection cassette were conducted in the SW102 strain (Warming *et al.*, 2005). The KSHV BAC36 transformed the modified DH10B strain SW102, and the resultant colonies, under chloramphenicol selection, were screened for clones carrying an intact KSHV BAC36 using *Sac*I digestion. Interestingly *Sac*I digestion revealed that KSHV BAC36s were severely deformed in all screened SW102 clones, indicating that SW102 was not compatible with KSHV BAC36 (Supplement data Fig. S1). Both results suggested that KSHV BAC36 was too unstable to introduce a site-directed point mutation into the viral genome using the Counter-Selection BAC Modification Kit. To address this concern, KSHV BAC36 was modified to make the BAC stable during the entire Red/ET recombination process. The Amp resistance (Amp^r) gene was amplified from the pGEM-T vector and introduced into the KSHV genome at 117646 through Red/ET recombination following the Amp^r selection (Fig. 1B). The Amp^r gene was inserted right opposite the site where the choramphenicol resistance (Chl^r) gene is located on KSHV BAC36; the Chl^r gene was included in the Hyg/BAC/GFP cassette. The Amp^r

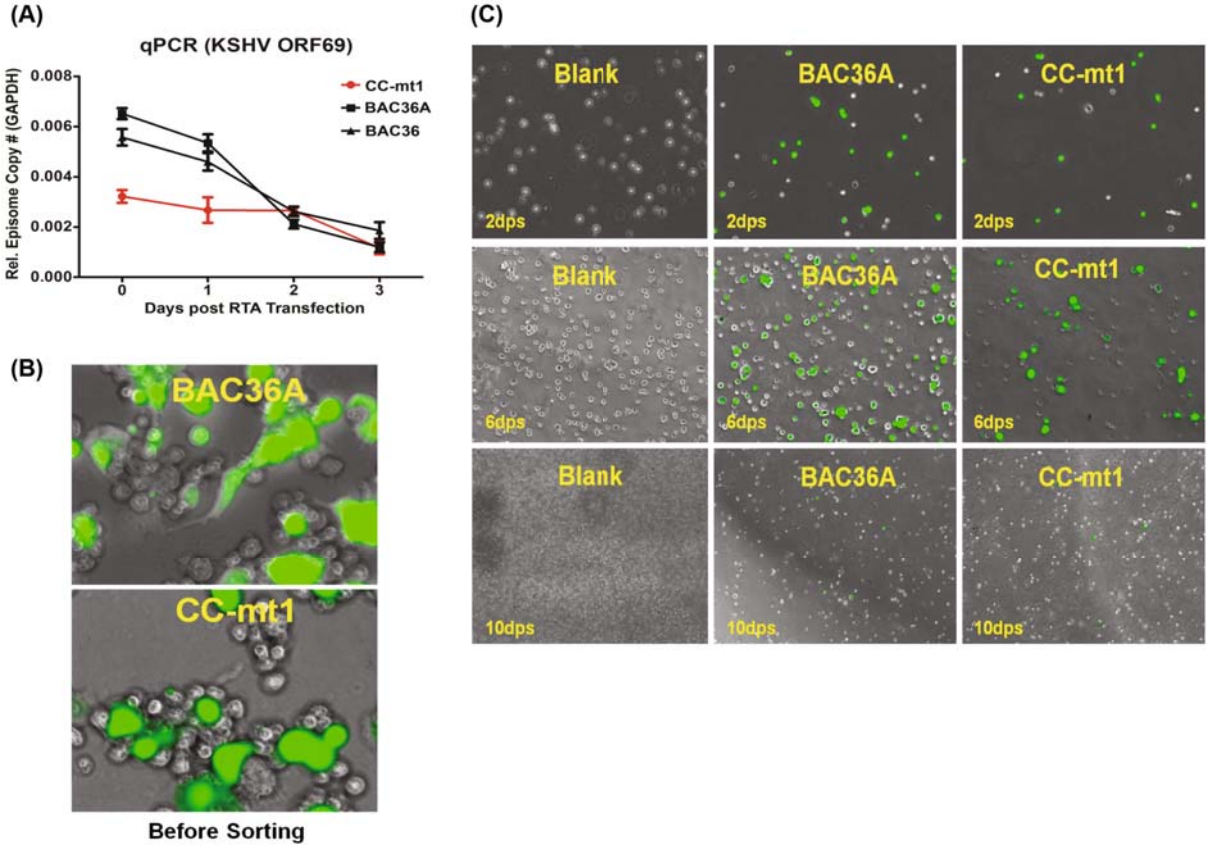

Fig. 2. KSHV infection of B-cell lymphoma using coculturing systems. (A) RTA induction reduced the copy numbers of KSHV episomes in 293T-KSHV cells: Stable 293T cell lines carrying either KSHV parental BAC36 (293T-BAC36), BAC36A (293T-BAC36A) or mutant BAC (293T-CC-mt1) were transfected with RTA overexpression constructs to induce KSHV lytic reactivation. The RTA transfected 293T-KSHV cells were harvested at designated periods, subjected to DNA extraction and copy numbers of KSHV episomes relative to GAPDH were determined. (B) BJAB and 293T-KSHV cells maintained their intact morphologies during coculture for 48 h in DMEM medium: RTA overexpressing plasmids were transfected into 2×10^6 293T-KSHV cells to induce viral lytic replication. At 2 days post-induction we overlaid 4×10^6 BJAB cells onto RTA induced 293T-KSHV cells, which started to infect BJAB cells by KSHV virions produced from 293T-KSHV cells. (C) Coculturing induced KSHV virions derived from 293T-KSHV cells to infect KSHV negative BJAB: At 2 days post-coculturing, most BJAB cells suspended in DMEM medium were collected and subjected to immunostaining with anti-CD45 antibody. We then sorted the GFP positive and CD45 positive BJAB cells using a FACs Aria III (BD Science). The sorted GFP+ and CD45+ cells were considered as KSHV-infected BJAB cells, which we further cultured up to 10 days post-sorting.

gene was 84451 bps from the Hyg/BAC/GFP cassette inserted at bp 33195 of the KSHV genome. The opposite location of the Amp^r gene was expected to increase the fidelity of selection for intact KSHV BAC36. The Amp^r gene insertion was confirmed by PCR and sequencing (Fig. 1C), and Bacmid integrity was confirmed by *Sac*I digestion (Fig. 1D). PCR revealed the presence of a 906-bp Amp^r gene in the KSHV BAC36A genome that is not present in BAC36. The *Sac*I restriction site on the KSHV genome at 127394 was removed by a LoxP insertion at the CTCF/Cohesin binding sites (KSHV genome 127376–127529). Thus, a distinct 4.8-kb DNA fragment (marked by red arrow) disappeared when the KSHV mutant BAC36s carrying mutations in the CTCF/Cohesin binding sites were digested by *Sac*I restriction enzyme. This feature was used to either screen the CTCF/Cohesin mutant BAC from parental BAC36 or check KSHV BAC36 stability in the DH10B strain. The insertion of the Amp^r gene into BAC36 did not cause any detectable deformation or additional recombination. The resultant BAC36 inserted with Amp^r gene was named BAC36A. Furthermore, any effect of Amp^r gene insertion on production of KSHV ORFK12 and ORF69 transcripts was tested by RT-qPCR. The production of KSHV ORFK12 and ORF69 transcripts were compared in 293T stable cells carrying either BAC36 or BAC36A. We could not detect any significant differences in transcription levels of ORFK12 and ORF69 (Fig. 1E) between BAC36 and BAC36A, respectively. Next, the rpsL-neo cassette was PCR amplified to construct CC-mt1 that flanked 50 bp on both sides of KSHV genome position 127348 (downstream of ORF73) and KSHV genome position 127833 (upstream to ORF73). The resulting rpsL-neo cassette was then introduced into KSHV BAC36 to replace KSHV 127348-127833 through Red/ET recombination. DNA fragments spanning mutated CTCF/Cohesin binding sites in the KSHV latency control region (KSHV genome 127376–127529) were PCR amplified as previously described (Kang and Lieberman, 2009). These DNA fragments were recombined into KSHV CTCF/Cohesin binding sites to replace the rpsL-neo cassette by Red/ET recombination counter-selection against streptomycin. Intriguingly, the Red/ET recombination using BAC36A produced KSHV intact Bacmids that did contain targeted point mutations in the CTCF/Cohesin binding sites without any deformation or additional recombination (Fig. 1F). We screened fewer than 30 clones and successfully obtained 29 clones that produced no deformation in Bacmid integrity. The resulting KSHV BAC named CC-mt1 was transfected into 293T cells, and 293T cells carrying CC-mt1 were selected for two weeks under hygromycin B in parallel with BAC36 and BAC36A as controls. Additionally RT-qPCR was conducted to test if the Amp^r gene insertion caused any defects in producing KSHV ORFK12 and ORF69 transcripts (Fig. 1E). The transcriptional levels of KSHV ORFK12 and ORF69 were compared between the CTCF/cohesin binding site mutant BAC (CC-mt1) and parental KSHV BACs such as BAC36 and BAC36A. We could

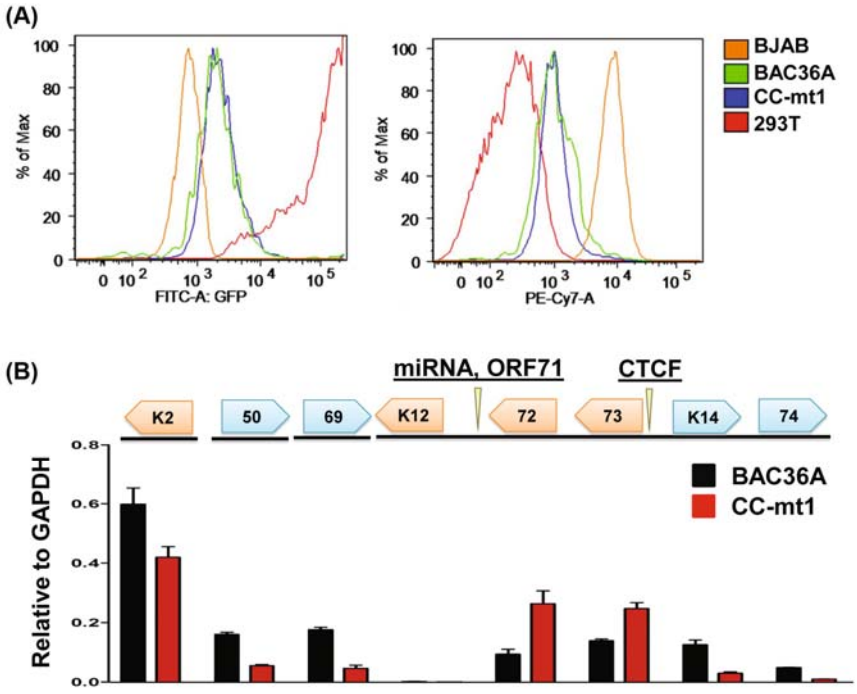

Fig. 3. Confirmation of BJAB infected by KSHV. (A) BJAB-KSHV cells demonstrated GFP$^+$CD45$^+$ phenotypes: Coculturing was conducted with BJAB cells and stable 293T-KSHV cells carrying either BAC36A or CC-mt1. The KSHV episome in BJAB cells was maintained until 8 days post-infection (6 days post-sorting). We reconfirmed that GFP (FITC staining, left panel) and CD45 (PE-Cy7 staining, right panel) were positive in BJABs 6 days post-sorting. (B) Depletion of CTCF-Cohesion binding to the KSHV latency control region caused upregulation of KSHV major latent transcripts: RT-qPCR was used to determine transcription profiles of KSHV latent and lytic genes in KSHV infected BJAB (6 days post-sorting).

not detect any significant differences in transcripts of ORFK12 and ORF69 (Fig. 1E) between BAC36 and BAC36A or CC-mt1 in 293T cells. Taken together, our data show that the insertion of the Amp^r gene did not cause any defects by producing neighboring gene transcripts, but it significantly enhanced the fidelity of target homologous recombination.

KSHV virions reactivated from 293T cells transfected with KSHV BAC36 derivatives enabled infection of BJAB cells

In order to induce KSHV lytic replication from 293T cells carrying KSHV BAC36 (293T-BAC36) or CC-mt1 (293T-CC-mt1), we transfected these 293T cells with RTA overexpression constructs and induced lytic replication for 48 h. While copy numbers of extracellular viral episomes significantly increased (data not shown, Kang *et al*., 2011), RTA transfection decreased copy numbers of intracellular viral episomes in the 293T cells (Fig. 2A). These changes in KSHV episome copy numbers suggested that KSHV virions were produced inside 293T-BAC36 or 293T-CC-mt1 cells, and then relocated into the extracellular medium. To infect with KSHV virions, BJAB (KSHV-EBV negative Burkitt-like B-cell lymphoma) cells were then overlaid onto the RTA transfected 293T cells (293T-KSHV(BAC36 or CC-mt1)-RTA) and cocultured for 48 hours. Interestingly, there were not any detectable morphological changes in BJAB cells even though they were cultured in 10% FBS supplemented DMEM medium for 48 h (Fig. 2B). After gentle shaking to make BJAB cells float, DMEM medium with floating BJAB cells was collected and then the cells were pelleted for harvesting. Following staining with PE-Cy^{TM} 7 Mouse Anti-Human CD45, the CD45$^+$ and GFP$^+$, BJAB cells were positively selected by FACS sorting and further cultured in FBS supplemented RPMI medium for an additional 10 days. GFP$^+$ BJAB cells were clearly observed at 2 days post-sorting (2 dps), reached maximum concentrations 6 days post-sorting (6 dps), but then disappeared 10 days post-sorting (10 dps, Fig. 2C), meaning that the BJAB cells maintained KSHV genomes only for a short period. This result demonstrated that KSHV virions derived from KSHV BAC36 or CC-mt1 enabled infection of B-cell lymphoma cells during coculturing.

Phenotypes caused by a KSHV CTCF mutation appeared similar for BJAB and 293T cells

Since the population of the GFP$^+$ BJAB cells reached its maximum ~ 6 dps, the BJAB cells at this time were re-subjected to FACS analysis to confirm their properties. The GFP$^+$ CD45$^+$ feature in the BJAB cells were clearly observed for 293T-KSHV and BJAB cells by either GFP or PE-Cy7 subset FACs analysis (Fig. 3A). We also tested whether KSHV gene expression patterns in GFP$^+$ BJAB cells were similar to 293T-KSHV cells using RT-qPCR. Point mutations in KSHV CTCF/Cohesin binding sites appeared to upregulate major latent transcripts such as LANA, whereas the mutation downregulated KSHV lytic gene expression (Fig. 3B). This transcription pattern in GFP$^+$ BJAB cells was consistent with those observed in the 293T-KSHV cells (Kang *et al*., 2011).

Discussion

There are only a few studies that reported KSHV infection in primary B lymphocytes (Rappocciolo *et al*., 2008; Hassman *et al*., 2011; Myoung and Ganem, 2011a). One study used peripheral blood B cells for KSHV infection, while another used primary tonsillar lymphoid cells. These studies were evaluated in a search to overcome the difficulty of infecting B lymphocytes with KSHV. However, these studies have several limitations in that it was difficult to obtain human primary lymphocyte samples, isolate primary B lymphoid cells, and secure B lymphoid cells long enough to do biochemical analyses. Thus, it was necessary to develop another KSHV B lymphocyte infection system that allows easy access to reagents. If the KSHV B-cell infection system is optimized to apply the KSHV BAC36 reverse genetic system, the infection system would be one of the best tools for studying KSHV biology in B-cells because only KSHV BAC36 is available for genetic modification in the KSHV genome. However, no one to date has shown that KSHV virions derived from KSHV BAC36 infect B-cell lymphocytes other than a few adherent cells, including 293 and HUVEC cells, among others (Zhou *et al*., 2002; Gao *et al*., 2003; Qian *et al*., 2007). Furthermore, recent sequencing of the complete KSHV BAC36 genome revealed that a 9-kb LUR fragment in the terminal repeat region was duplicated in the BAC36 genome (Yakushko *et al*., 2011). This duplication made it complicated to determine why KSHV BAC36-derived virions did not successfully infect any B lymphocytes. Besides the difficulty of obtaining KSHV B-cell infection, it requires laborious work to introduce site-directed point mutations into KSHV BAC36 by homologous recombination. Few studies have demonstrated a successfully introduced point mutation into the KSHV genome for KSHV BAC36. In our case, we screened more than 500 clones but didn't find even a single KSHV BAC36 clone carrying the targeted point mutations. Most clones derived from two distinct homologous recombination processes appeared to contain severely deformed KSHV genomes, suggesting that KSHV BAC36 is not sufficiently stable to avoid deformation during homologous recombination. To address these concerns and overcome current impediments, our study provided a new KSHV B-cell infection model equipped with both the KSHV BAC36A system and the coculturing-based BJAB infection.

Our study provides a new approach that overcomes a couple of barriers intrinsic to the KSHV BAC36 reverse genetic system and KSHV B cell infection. First, simple modification of KSHV BAC36 (named as BAC36A) significantly enhanced the fidelity and yield of gene-targeted site-directed mutagenesis. The KSHV genome size has been estimated to be 140 kb to 165 kb in previous reports (Russo *et al*., 1996). The hygromycin/BAC/GFP cassette containing the chl^r gene was inserted at genome bp 33195 to construct KSHV BAC36, whereas the Amp^r gene was located at KSHV genome bp 117646. Thus, these two antibiotic resistance genes are located at opposite ends of the KSHV genome, suggesting that two antibiotics exert high pressure against BAC36A being deformed and select viral bacmids with integrity during a series of homologous recombinations. Consequently, BAC36A will facilitate the introduction of

point mutations into the KSHV genome. Second, a coculture system allowed successful KSHV infection of the B cell lymphoma cell line BJAB, which was mediated by KSHV virions derived from modified KSHV BAC36A. Interestingly, transcriptional defects caused by mutations in the CTCF/Cohesin binding sites during KSHV latent/lytic gene expression appeared to be similar for GFP^+ BJAB cells and 293T-KSHV cells. This result suggests that the physical CTCF/Cohesin binding is more critical in regulating KSHV latent/lytic gene expression than cofactors that may exist distinctively from other cell lines. Although the cocultre system that allowed KSHV B-cell infection needs some optimization, it will eventually improve the KSHV-BAC36 reverse genetic system in B lymphocytes.

Despite the advantages, there are a couple of concerns for use of the KSHV BAC36A and coculture system as described above. First, the BJAB cell line did not maintain the KSHV genome for long periods, for example up to 8 days post-infection. This suggests that cofactors absent in BJAB but present at primary effusion lymphomas (PEL) may be required to maintain the KSHV genome or establish KSHV latency. Such cofactors are left to be determined for developing more advanced KSHV reverse genetic systems and B-cell infection models. In addition to BJAB, the short period of KSHV genome maintenance was observed in most primary cells including HUVEC and tonsillar lymphoid cells (Hassman *et al*., 2011; Myoung and Ganem, 2011a). Thus, it is also possible that long-term KSHV genome maintenance requires specific cellular activation or differentiation by counterpart cells such as effector T cells that activate B cells. Further, significant loss of CD45 was observed when KSHV virions infected BJAB cells, indicating that BJAB cells lose their B-cell properties. CD45 is a high molecular weight transmembrane protein with intrinsic phosphatase activity that plays an essential role in regulating the B-cell antigen receptor-mediated activation (Hermiston *et al*., 2009). KSHV produces a large number of proteins that antagonize the immune system of its host (Coscoy, 2007). These viral immunomodulators deregulate the innate and adaptive immune responses. Among the KSHV immunomodulators, K15 is known to inhibit B-cell signaling and likely to involve downregulation of CD45 expression in host cells infected with KSHV (Pietrek *et al*., 2010). Further study is needed to define the mechanism by which KSHV infection leads the loss of CD45. However, there are several limitations in the BAC36A BJAB infection model, and our new KSHV infection system may contribute to elucidation of the mechanisms of both viral transmission and tumorigenesis in B cells.

One interesting recent report provided a new approach for infecting a BJAB cell line with KSHV.219 (rKSHV.219) using a coculture system (Myoung and Ganem, 2011b). This coculture system seems to be similar to the coculture procedures that we used to infect BJAB with KSHV virions derived from KSHV BAC36A. However, KSHV BAC36A has one great advantage in that it allows us to introduce point mutations into the KSHV genome, unlike rKSHV.219. The principal significance of the current study is that it demonstrates a new method that enables one to infect B-cell lymphoma with KSHV virions derived from KSHV BAC36A.

Acknowledgements

We sincerely thank Dr. Paul Lieberman for providing essential help in designing the overall study plan, significant comments on the analysis of the data and a critical review of the manuscript. This research was supported by Kyungpook National University Research Fund, 2011.

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