Originally published as:

Budt, M., Hristozova, T., Hille, G., Berger, K., Brune, W.
Construction of a lytically replicating Kaposi's sarcoma-associated herpesvirus
(2011) Journal of Virology, 85 (19), pp. 10415-10420.

DOI: 10.1128/JVI.05071-11

Construction of a lytically replicating Kaposi's sarcoma-associated herpesvirus (KSHV)

Matthias Budt[1], Tsvetana Histozova[1], Georg Hille[1], Katrin Berger[1], and Wolfram Brune[1,2]

[1]Division of Viral Infections, Robert Koch Institute, Nordufer 20, 13353 Berlin,Germany
[2]Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Martinistr. 52, 20251 Hamburg, Germany

Corresponding author:
Phone: +49 40 48051351
Fax: +49 40 48051352
Email: wolfram.brune@hpi.uni-hamburg.de

Abstract

KSHV is found predominantly in a latent state in most cell types, impeding investigations of the lytic replication cycle. Here we engineered the cloned KSHV genome, BAC36, to enforce constitutive expression of the main lytic switch regulator, RTA (ORF50). The resulting virus, KSHV-lyt, activated by default the lytic cycle and replicated to high titers in various cells. Using KSHV-lyt we showed that ORF33 (encoding a tegument protein) is essential for lytic KSHV replication in cell culture, but ORF73 (encoding the latent nuclear antigen, LANA) is not. Thus, KSHV-lyt should be highly useful to study viral gene function during lytic replication.

KSHV (human herpesvirus 8) is the etiologic agent of Kaposi's sarcoma and the B-cell-derived malignancies multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL) (3, 5). KSHV infects a variety of cell types *in vivo,* but predominantly establishes a latent state of infection (1). During latency, the viral genome is maintained in the nucleus as an episome, and only few viral genes are expressed. Although latent infection seems to be sufficient to promote cellular transformation, lytic replication is also required for KS development (17) and for virus dissemination. The switch from latency to lytic replication is initiated by the ORF50-encoded replication and transcription activator (RTA) protein. The ORF50 transcript consists of two exons separated by an intron, which encodes on the complementary strand ORF49 (Fig. 1A). Splicing of ORF50 mRNA allows the expression of RTA, which functions as an immediate-early transcription factor capable of inducing many viral and cellular genes (9, 15, 20). RTA expression is both necessary and sufficient to induce the complete progression of KSHV through the lytic cycle (9).
In vitro, KSHV infection is either abortive or results in latency in most cell types (1). Lytic replication was found to some extent after infection of primary human endothelial cells, but was not self-sustaining over longer periods of time (4, 7). In latently-infected cells, KSHV reactivation can be triggered by chemical inducers such as 12-O-tetradecanoylphorbol-13-acetate (TPA) or sodium butyrate (13). However, these chemicals are quite toxic and can cause unwanted side effects (6). Alternatively, enforced RTA expression *in trans* by a plasmid or viral vector can be used to drive KSHV lytic replication (1). However, these systems are not self-sustaining, and replication stops when the inducing stimulus is withdrawn. Here we describe the construction and characterization of a recombinant KSHV carrying an intronless ORF50 gene under the control of a constitutively active promoter. The recombinant virus enters by default the lytic replication cycle in different cell types without the need for further induction. Infected cells display cytopathic effect (CPE), produce expanding foci, express all kinetic classes of viral genes, and release considerable amounts of virus progeny into the supernatant. We further show that

this lytically replicating KSHV can be used to analyze viral gene function during lytic replication.

A KSHV genome derived from the PEL cell line BCBL1 has been cloned as a bacterial artificial chromosome (termed BAC36) in *E.coli,* making it amenable to bacterial mutagenesis techniques (26). We used BAC36 to construct a lytically replicating KSHV clone. First we replaced ORF50 exon 1 and ORF49 (nt 71110 to 72093 in BAC36, GenBank accession# HQ404500) by homologous recombination using a zeocin resistance cassette PCR-amplified with primers 5'-CAACCTTACTCCGCAAGGGGTAGTCTGTTGTGAGAATACTGTCCAGGCAGTCAAGTCCT GCTCCTCCTCGGCCA-3' and 5'-CCGAGAGGCCGACGAAGCTTTCCACACAGGACCGC CGAAGCTTCTTACCCTTGTTGACAATTAATCATCGGCA-3' essentially as described (21). The resulting BAC was termed KSHVA49 (Fig. 1A). For the second recombination step, a helper plasmid was constructed. It contained ORF49, a kanamycin resistence *(kan)* marker, and ORF50 exon 1, and 50 nt flanking sequences for homologous recombination. Briefly, two oligonucleotides (5'-GATCTCCACCATGGCGCAAGATGACAAGGGTAAGAAGCTTCGGCGGTCCTGTGTGGAA AGCTTCGTCGGCCTCTCGGGCC-3' and 5'-GGCCGCAACCTTACTCCGCAAGGGGTAG TCTGTTGTGAGAATACTGTCCAGGCAG-3') were inserted into pReplacer (11) between the *BglII* and *ApaI* sites and *NotI* and EcoRI sites, respectively. Then, ORF49 was PCR-amplified and inserted between BamHI and EcoRI sites. A *kan* marker flanked by FLP recombination target (FRT) sites was introduced at the *Eco*RI site. The recombination cassette was excised with *Not*I and *Apa*I from the helper plasmid and used to modify KSHVA49 (Fig. 1A). Finally, the *kan* marker was removed with FLP recombinase yielding KSHV-lyt, in which an intronless ORF50 gene is driven by a cellular phosphoglycerate kinase (pgk) promoter. All constructs were analyzed by restriction digest and gel electrophoresis (Fig. 1B), by PCR, and by sequencing the relevant regions (data not shown).

Transfection of BAC DNA into telomerase-immortalized retinal pigment epithelial cells (hTERT-RPE1, ATCC CRL-4000) was monitored by expression of GFP, which is encoded adjacent to the BAC cassette (26). Transfection of KSHV-lyt resulted in the development of morphologically distinct foci of GFP-expressing cells (Fig. 2A), suggesting that KSHV-lyt-derived virus was able to cause CPE and spread to neighboring cells without any further exogenous stimulus. Foci increased in size and number over time until the entire monolayer was infected. By contrast, the parental BAC36 did not spread and did not form foci. After transfer of supernatant from infected cells to fresh RPE1 cells, new foci appeared, even if the supernatant was passed through a 0.45 urn filter.

By the same three-step procedure outlined in figure 1A we also constructed KSHV-lyt[-49]. ORF49 was not re-inserted in the second recombination step, but otherwise KSHV-lyt[-49] was constructed analogously to KSHV-lyt. KSHV-lyt[-49] formed foci and replicated in RPE1 like KSHV-lyt (data not shown), indicating that ORF49 is not required for KSHV lytic replication. However, since a previous study has shown that ORF49 cooperates with RTA to activate several KSHV lytic promoters (8), we decided to continue further work only with KSHV-lyt.

Surprisingly, serial passaging of KSHV-lyt virus resulted in a loss of GFP expression within the first 2-3 passages (Fig. 2B). KSHV virion DNA was recovered from supernatants of infected RPE1 cells and analyzed by restriction digest. Compared to KSHV-lyt BAC DNA, the virion DNA lacked the region containing the BAC cassette (Fig. 2C, D), suggesting that the BAC replicon and the adjacent GFP expression cassette were lost during virus reconstitution and lytic replication. In an attempt to avoid the loss of GFP expression, we constructed a modified version of KSHV-lyt, in which the BAC cassette contained a Cre recombinase gene and was flanked with loxP sites to make it self-excising. Such a procedure has been used previously for other herpesvirus BACs (19, 24). The GFP gene was kept outside of the loxP-flanked region in order to preserve GFP expression after excision of the BAC replicon. Much to our chagrin, this modification did not prevent loss of GFP expression during virus reconstitution (data not shown). When we further investigated

the underlying reason for this phenomenon, we found out that the region, in which the BAC cassette was inserted, was duplicated in the parental BAC36. The second copy of the region ranging approximately from ORF K5 to ORF19 is located within the terminal repeats (TRs) and contains the BAC cassette (data not shown). While this work was in progress, an analysis of the complete BAC36 sequence was published by others (23). The paper identified and documented the same duplication that we had observed. Hence, we did not further investigate this property of BAC36. However, the presence of the BAC cassette within the duplicated region within the TR region offers a rational explanation for the rapid loss of the BAC cassette and the GFP gene. In fact, a recent publication suggested that the TRs are a suitable location for insertion of the BAC cassette because it will be automatically excised upon virus reconstitution by terminal repeat-mediated homologous recombination (25).

RPE1 cells were used routinely for virus reconstitution from BAC DNA and for titration of infectious KSHV. They have an intact contact inhibition response and tolerate the prolonged culturing required to recover replicating virus after BAC transfection. RPE1 cells were also the preferred cell type for titration because they developed the clearest CPE and showed most reliable and reproducible results. Viral titers (focus-forming units) were determined using the median tissue culture infective dose (TCID50) method (16). Larger quantities of infectious virus were grown on RPE1 cells and harvested from the supernatants of infected cells. High-titer KSHV-lyt stocks were obtained by pelleting the virus (180 min at 27000 g) and resuspending it in a smaller volume of complete medium. To determine the replication capability of KSHV-lyt, growth kinetics were determined on different cells. RPE1, Vero (ATCC CRL-1587D) and primary HUVEC (Lonza, Switzerland) were infected at an MOI of 0.02 using 5 ug/ml polybrene and centrifugal enhancement of infection (30 min at 900 *g).* On all cell lines used here, KSHV-lyt infection lead to cell rounding and swelling, release of infectious virions into the supernatant, and finally cell demise several days after infection. After low-MOI infection, the virus replicated to maximum titers of about 10^5 TCID50/ml in RPE1 cells.

Next we tested whether KSHV-lyt can be used to determine the importance of viral genes for lytic replication. First we replaced ORF33, encoding a tegument protein (27) by a *galK/kan* cassette with methods described previously (18). In a second step, *galK/kan* was removed by homologous recombination using a synthetic oligonucleotide (5'-GCTATAGGGCGTCGAAGGAGGATCTGGTGTTCATTCGAGGCCGCT ATGGCTAGCAGCATGTTGCGCACATCAGCGAGCTGGACCGTCCTCCGGGTCGCGT-3') generating the seamless deletion mutant KSHV-lytA33. The revertant virus, Rev33, was obtained by replacing *galK/kan* with a PCR-amplified ORF33 sequence (Fig. 3A). Mutant BACs were checked by restriction digest (Fig. 3B) and transfected into RPE1 cells (Fig. 3C). As expected, both parental and revertant BACs yielded infectious virus that replicated to comparable titers (Fig. 3D). The A33 deletion mutants (with or without *galK/kan)* repeatedly failed to reconstitute infectious virus, indicating that ORF33 is essential for lytic replication. This result matches similar findings in the related y-herpesvirus MHV-68, for which ORF33 is also essential for replication (10).

The LANA-encoding ORF73 was deleted by a similar approach (Fig. 4A). ORF73 was first replaced with *galKlkan,* yielding KSHV-lytA73. Then, a cloned KSHV fragment containing ORF73 and approximately 150 nt of flanking KSHV sequence (kindly provided by Rolf Renne) was used to generate the revertant Rev73. LANA expression from the recombinant KSHVs was checked by immunofluorescence (Fig. 4B) and immunoblot (Fig. 4C). As the cloned ORF73 sequence used for Rev73 was derived from a different KSHV clone and contained fewer internal repeats, Rev73-expressed LANA was slightly smaller in size than that of the parental virus. All three recombinant viruses expressed RTA and K8.1 proteins in infected RPE1 cells (Fig. 4C), demonstrating that LANA expression is not essential for KSHV lytic replication. However, the A73 mutant grew to slightly lower titers than the parental and revertant viruses, indicating that LANA might contribute directly or indirectly to lytic virus production (Fig. 4D). In contrast, LANA-deficient KSHV BAC36 and Rhesus Rhadinovirus showed increased virus production in previous studies, which was

attributed to the loss of LANA-mediated repression of the RTA promoter (14, 22). This repression is probably absent in KSHV-lyt, as RTA expression is driven by a heterologous promoter.

In summary, we demonstrated that a molecular KSHV clone modified to express RTA constitutively replicates to substantial titers in cultured cells. The lytically replicating KSHV is well-suited to study viral gene function during lytic replication. Another promising application for lytically replicating KSHV is the field of antiviral drug testing. Most of the available antiherpesviral drugs target the lytic replication phase (12, 17) and are, therefore, difficult to test with previously available systems in which KSHV is predominantly latent (2). Of course, PEL cell lines and unmodified BAC36 remain the systems of choice for investigating latency and reactivation.

We thank Shou-Jiang Gao for the BAC36, Gary Hayward and Keji Ueda for antibodies, Thomas Schulz and Cornelia Henke-Gendo for sharing the BAC36 sequence before publication, and Antonio Gallo and Adam Grundhoff for a critical reading of the manuscript.

References

1. **Bechtel, J. T., Y. Liang, J. Hvidding, and D. Ganem.** 2003. Host range of Kaposi's sarcoma-associated herpesvirus in cultured cells. J Virol **77:**6474-81.
2. **Casper, C., and A. Wald.** 2006. The Use of Antiviral Drugs in the Prevention and Treatment of Kaposi Sarcoma, Multicentric Castleman Disease and Primary Effusion Lymphoma, p. 289-307, Current Topics in Microbiology and Imunology, vol. 312. Springer Verlag, Berlin.
3. **Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore.** 1994. Identification of herpesvirus-like DNA sequences in AIDS associated Kaposi's sarcoma. Science **266:**1865-9.
4. **Ciufo, D. M., J. S. Cannon, L. J. Poole, F. Y. Wu, P. Murray, R. F. Ambinder, and G. S. Hayward.** 2001. Spindle cell conversion by Kaposi's sarcoma-associated herpesvirus: formation of colonies and plaques with mixed lytic and latent gene expression in infected primary dermal microvascular endothelial cell cultures. J Virol **75:**5614-26.
5. **Damania, B.** 2004. Oncogenic gamma-herpesviruses: comparison of viral proteins involved in tumorigenesis. Nat Rev Microbiol **2:**656-68.
6. **Filippovich, I., N. Sorokina, K. K. Khanna, and M. F. Lavin.** 1994. Butyrate induced apoptosis in lymphoid cells preceded by transient over-expression of HSP70 mRNA. Biochem Biophys Res Commun **198:**257-65.
7. **Gao, S. J., J. H. Deng, and F. C. Zhou.** 2003. Productive lytic replication of a recombinant Kaposi's sarcoma-associated herpesvirus in efficient primary infection of primary human endothelial cells. J Virol **77:**9738-49.
8. **Gonzalez, C. M., E. L. Wong, B. S. Bowser, G. K. Hong, S. Kenney, and B. Damania.** 2006. Identification and characterization of the Orf49 protein of Kaposi's sarcoma-associated herpesvirus. J Virol **80:**3062-70.
9. **Gradoville, L., J. Gerlach, E. Grogan, D. Shedd, S. Nikiforow, C. Metroka, and G. Miller.** 2000. Kaposi's sarcoma-associated herpesvirus open reading frame 50/Rta protein activates the entire viral lytic cycle in the HH-B2 primary effusion lymphoma cell line. J Virol **74:**6207-12.
10. **Guo, H., L. Wang, L. Peng, Z. H. Zhou, and H. Deng.** 2009. Open reading frame 33 of a gammaherpesvirus encodes a tegument protein essential for virion morphogenesis and egress. J Virol **83:**10582-95.
11. **Jurak, I., and W. Brune.** 2006. Induction of apoptosis limits cytomegalovirus cross species infection. EMBO J **25:**2634-42.
12. **Kedes, D. H., and D. Ganem.** 1997. Sensitivity of Kaposi's sarcoma-associated herpesvirus replication to antiviral drugs. Implications for potential therapy. J Clin Invest **99:**2082-6.
13. **Lagunoff, M., J. Bechtel, E. Venetsanakos, A. M. Roy, N. Abbey, B. Herndier, M.**

McMahon, and D. Ganem. 2002. De novo infection and serial transmission of Kaposi's sarcoma-associated herpesvirus in cultured endothelial cells. J Virol **76:**2440-8.
14. **Li, Q., F. Zhou, F. Ye, and S. J. Gao.** 2008. Genetic disruption of KSHV major latent nuclear antigen LANA enhances viral lytic transcriptional program. Virology **379:**234-44.
15. **Lukac, D. M., J. R. Kirshner, and D. Ganem.** 1999. Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. J Virol **73:**9348-61.
16. **Mahy, B. W. J., and H. O. Kangro.** 1996. Virology methods manual. Academic Press, San Diego, CA.
17. **Martin, D. F., B. D. Kuppermann, R. A. Wolitz, A. G. Palestine, H. Li, and C. A. Robinson.** 1999. Oral ganciclovir for patients with cytomegalovirus retinitis treated with a ganciclovir implant. Roche Ganciclovir Study Group. N Engl J Med **340:**1063-70.
11
18. **Qian, Z., B. Xuan, T. T. Hong, and D. Yu.** 2008. The full-length protein encoded by human cytomegalovirus gene UL117 is required for the proper maturation of viral replication compartments. J Virol **82:**3452-65.
19. **Smith, G. A., and L. W. Enquist.** 2000. A self-recombining bacterial artificial chromosome and its application for analysis of herpesvirus pathogenesis. Proc Natl Acad Sci U S A **97:**4873-8.
20. **Sun, R., S. F. Lin, L. Gradoville, Y. Yuan, F. Zhu, and G. Miller.** 1998. A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. Proc Natl Acad Sci U S A **95:**10866-71.
21. **Valchanova, R. S., M. Picard-Maureau, M. Budt, and W. Brune.** 2006. Murine cytomegalovirus m142 and m143 are both required to block protein kinase R mediated shutdown of protein synthesis. J Virol **80:**10181-90.
22. **Wen, K. W., D. P. Dittmer, and B. Damania.** 2009. Disruption of LANA in rhesus rhadinovirus generates a highly lytic recombinant virus. J Virol **83:**9786-802.
23. **Yakushko, Y., C. Hackmann, T. Gunther, J. Ruckert, M. Henke, L. Koste, K. Alkharsah, J. Bohne, A. Grundhoff, T. F. Schulz, and C. Henke-Gendo.** 2011. Kaposi's Sarcoma-Associated Herpesvirus Bacterial Artificial Chromosome Contains a Duplication of a Long Unique-Region Fragment within the Terminal Repeat Region. J Virol **85:**4612-7.
24. **Yu, D., G. A. Smith, L. W. Enquist, and T. Shenk.** 2002. Construction of a self excisable bacterial artificial chromosome containing the human cytomegalovirus genome and mutagenesis of the diploid TRL/IRL13 gene. J Virol **76:**2316-28.
25. **Zhou, F., Q. Li, S. W. Wong, and S. J. Gao.** 2010. Autoexcision of bacterial artificial chromosome facilitated by terminal repeat-mediated homologous recombination: a novel approach for generating traceless genetic mutants of herpesviruses. J Virol **84:**2871-80.
26. **Zhou, F. C., Y. J. Zhang, J. H. Deng, X. P. Wang, H. Y. Pan, E. Hettler, and S. J. Gao.** 2002. Efficient infection by a recombinant Kaposi's sarcoma-associated herpesvirus cloned in a bacterial artificial chromosome: application for genetic analysis. J Virol **76:**6185-96.
27. **Zhu, F. X., J. M. Chong, L. Wu, and Y. Yuan.** 2005. Virion proteins of Kaposi's sarcoma-associated herpesvirus. J Virol **79:**800-11.

Figures

Figure 1. Construction of KSHV-lyt. (A) Schematic illustration of KSHV BAC36 mutagenesis. ORF50 exon 1 and ORF49 were first replaced by a *zeo* cassette. Then, ORF49 was re-inserted in reverse orientation together with a pgk promoter and ORF 50 exon 1. The *kan* cassette flanked by FRT sites (black ovals) was subsequently removed with FLP recombinase. Nt positions refer to GenBank HQ404500. (B) XbaI restriction pattern of the parental and mutant BAC genomes in an ethidium bromide-stained agarose gel. For better visibility, images of the upper and lower parts of the gel were taken separately. Fragments affected by mutagenesis as predicted in panel A are indicated by arrow heads.

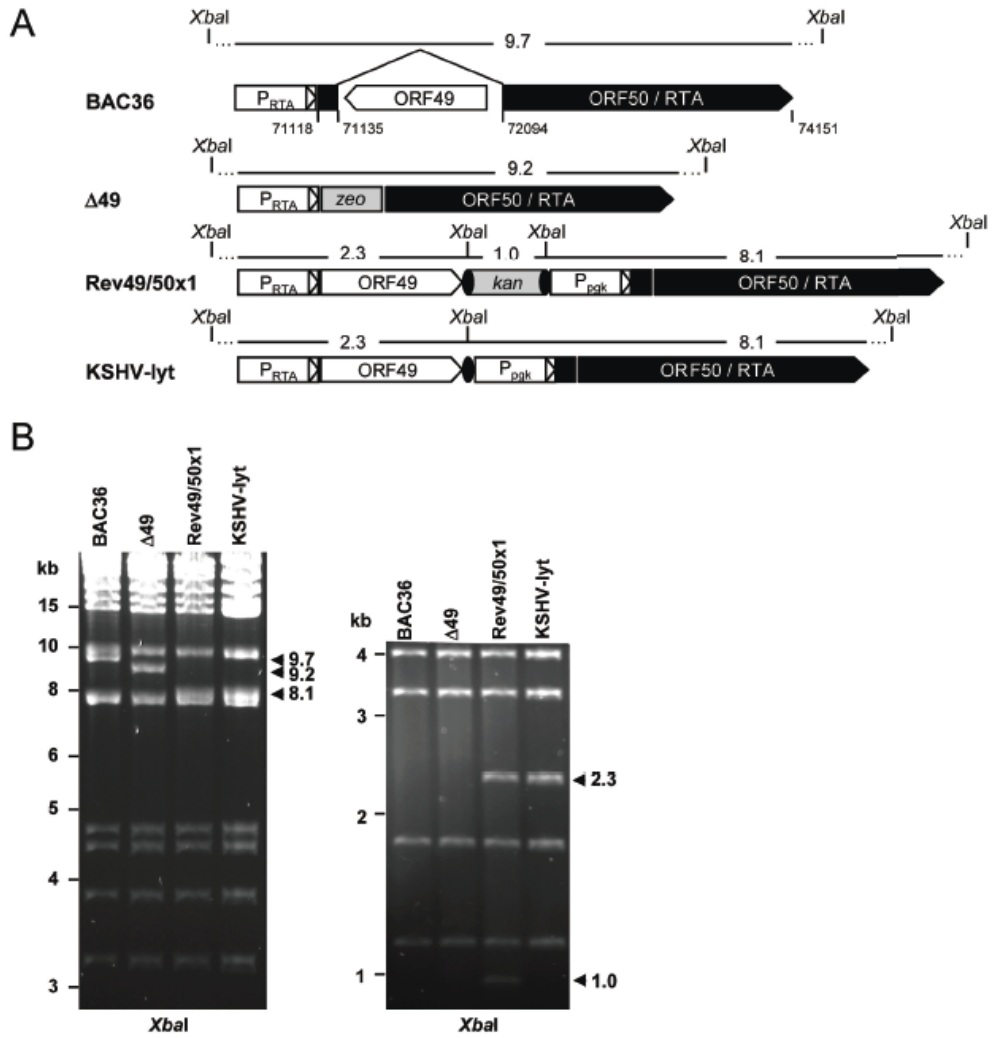

Figure 2. Analysis of KSHV-lyt replication. (A) Virus reconstitution following BAC transfection into RPE1 cells. GFP expression and focus formation was observed by fluorescence microscopy over time. Images were taken 2 and 8 days post transfection (dpt). (B) Focus formation 8 days after transfering infected cell supernatant onto fresh RPE1 cells. (C) Comparison of KSHV-lyt BAC and virion DNA. Equal amounts of DNA were digested with PmeI or *XhoI* and analyzed by gel electrophoresis. Fragments representing the BAC cassette are indicated by arrow heads. Asterisks indicate additional differences. (D) The PmeI and XhoI restriction maps with the expected fragments in the BAC cassette region are depicted. (E) Replication of KSHV-lyt on different cells after low MOI infection. Titers (focus-forming units) were determined on RPE1 cells using the $TCID_{50}$ method. DL, detection limit.

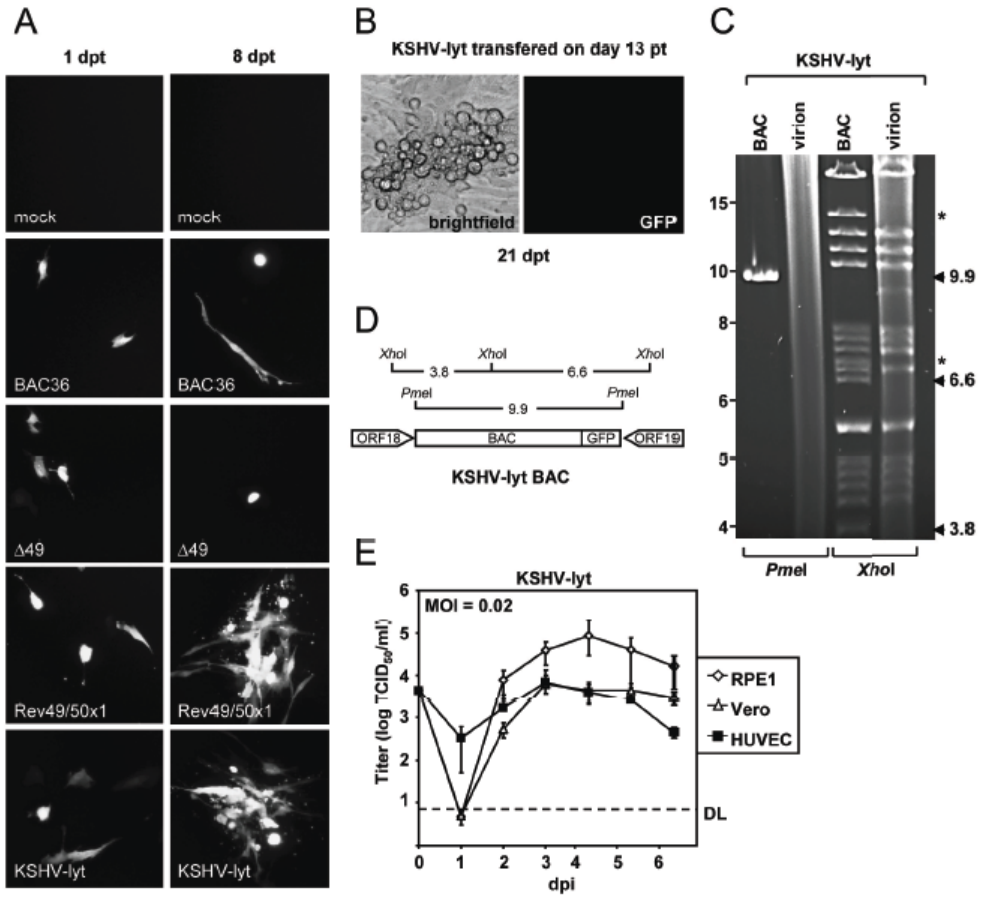

Figure 3. ORF33 is required for KSHV-lyt lytic replication. (A) Genomic organization of KSHV-lyt ORF33 mutants. Nt positions refer to GenBank HQ404500. (B) Acc65I (isoschizomer of KpnI) restriction pattern of the parental and mutant BAC genomes in an ethidium bromide-stained agarose gel. The relevant fragments (see panel A) are indicated by arrow heads. (C) RPE1 cells were transfected with recombinant BAC genomes as indicated, and GFP fluorescence was observed 10 days post transfection. (D) RPE1 cells were infected at an MOI of 0.02 TCID50/cell of the indicated viruses. Infectious virus (focus-forming units) released into the supernatants was titrated on RPE1 cells. DL, detection limit.

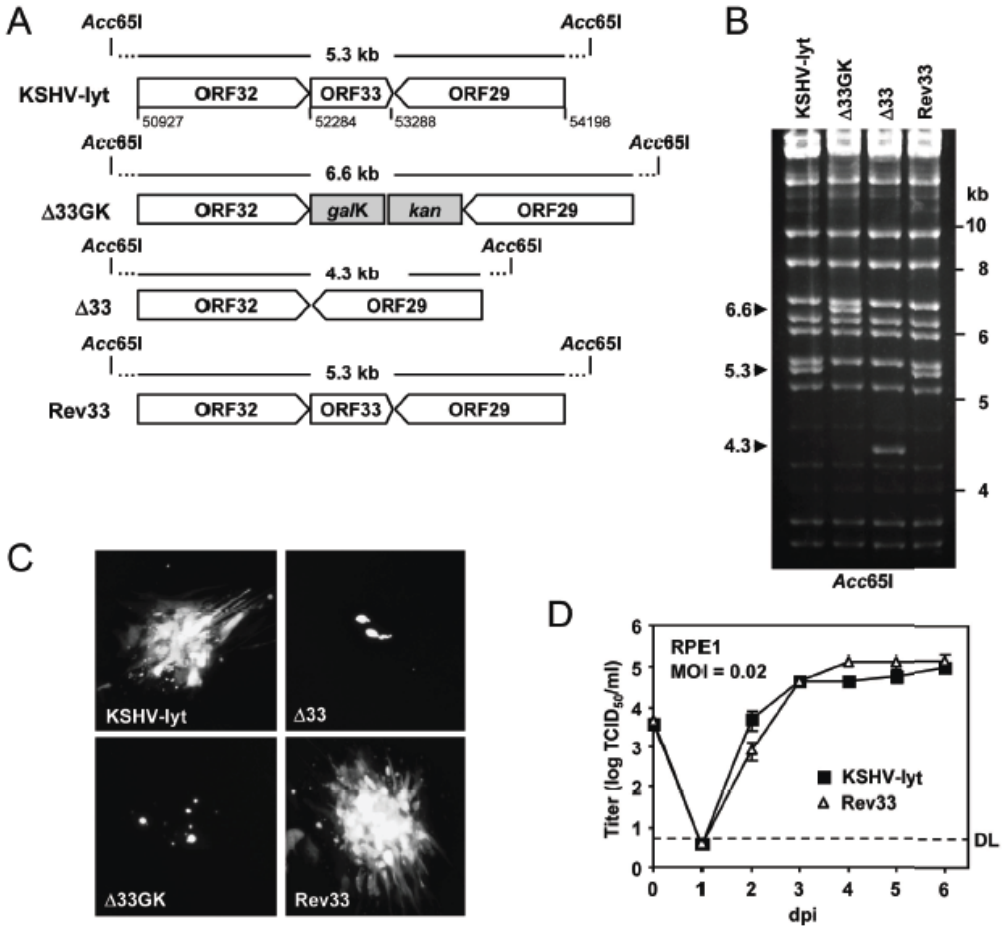

Figure 4. ORF73 is dispensable for KSHV-lyt lytic replication. (A) Genomic organization of KSHV-lyt ORF73 mutants. Nt positions refer to GenBank HQ404500. (B) RPE1 cells were infected as indicated and analyzed by immunofluorescence. RTA was stained with a rabbit antiserum (kindly provided by Gary Hayward), and AlexaFluor488-coupled anti-rabbit IgG (Invitrogen, Germany). LANA was visualized with mouse anti-LANA (Acris, Germany) and AlexaFluor568-coupled anti-mouse IgG. Nuclei were counterstained with DAPI. (C) Viral protein expression in infected RPE1 cells was analyzed by immunoblot with the same primary antibodies as above, or anti-K8.1 (Santa Cruz, CA) and peroxidase-coupled secondary antibodies (DAKO, Germany). (D) RPE1 cells were infected at an MOI of 0.005 TCID50/cell, and viral titers (focus-forming units) in the supernatant were determined. DL, detection limit.