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Identification of the Nuclear Export and Adjacent Nuclear Localization Signals for ORF45 of Kaposi's Sarcoma–Associated Herpesvirus

(Running title: NES and NLS of KSHV ORF45)

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ABSTRACT

Open reading frame 45 (ORF45) of Kaposi's sarcoma–associated herpesvirus 8 (KSHV) is an immediate early, phosphorylated, tegument protein and has been shown to play important roles at both early and late stages of viral infection. Homologues of ORF45 exist only in gamma herpesviruses, and the homology is limited. These homologues differ in their lengths of protein and subcellular localizations. We and others have reported that KSHV ORF45 is predominantly localized in the cytoplasm, whereas its homologue of murine herpesvirus 68 is exclusively localized in the nucleus. We observed that ORF45s of rhesus rhadinovirus and herpesvirus virus saimiri are exclusively in the nucleus. As a first step toward understanding the mechanism underlying the distinct intracellular distribution of KSHV ORF45, we identified the signals that control its subcellular localization. We found that KSHV ORF45 rapidly accumulated in the nucleus in the presence of lepotomycin B, an inhibitor of CRM1 (exportin 1)-dependent nuclear export, suggesting it could shuttle between nucleus and cytoplasm. Mutational analysis revealed that KSHV ORF45 contains a CRM1-dependent, leucine-rich-like nuclear export signal and an adjacent nuclear localization signal. Replacement of the key residues with alanines in these motifs of ORF45 disrupts its shuttling between cytoplasm and nucleus. The resulting ORF45 mutants have restricted subcellular localizations, either in the cytoplasm or in the nucleus exclusively. Recombinant viruses were reconstituted by introduction of these mutations into KSHV bacterial artificial chromosome BAC36. The resultant viruses have distinct phenotypes. A mutant virus in which ORF45 is restricted to the cytoplasm behaves as an ORF45-null mutant and produces 5- to 10-fold fewer progeny viruses than the wild type. In contrast, mutants in which ORF45 protein is mostly restricted to the nucleus produce numbers of progeny viruses similar to those produced by the wild type. These data suggest that the subcellular localization signals of ORF45 have important functional roles in KSHV lytic replication.

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INTRODUCTION

Kaposi's sarcoma–associated herpesvirus (KSHV) is a DNA tumor virus and the causative agent of several human cancers, including Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (3, 6). Like all herpesviruses, KSHV has two alternative life cycles: latent and lytic. During latency, only a few viral genes are expressed, and no progeny viruses are produced. Under appropriate conditions, latent viral genomes are activated, initiate lytic replication, and express a full panel of viral genes, a process that leads to viral assembly, release of progeny virus particles, and de novo infection of naïve cells (3, 6). KSHV establishes latent infection in the majority of infected cells in cases of KS, PEL, and MCD, but lytic replications occur in a small fraction. The recurrent and periodic lytic cycles of KSHV are believed to play critical roles in viral pathogenesis (6, 7).

ORF45 is a KSHV encoded gene product that plays a critical role in the viral lytic cycle. It is an immediate early protein and is also present in virus particles as tegument protein (26, 27, 30). Disruption of ORF45 has no significant effect on overall viral lytic gene expression or DNA replication in BAC36-reconstituted 293T cells induced with both TPA and sodium butyrate together. But the ORF45-null mutant produces 5- to 10-fold fewer progeny viruses than the wild type and the mutant virus has dramatically reduced infectivity, suggesting ORF45 plays important roles at both early and late stages of viral infection (29). In addition to its roles, as a tegument component, which are possibly involved in viral ingress and egress processes, KSHV ORF45 interacts with cellular proteins and modulates the cellular environment. At least two such functions have been described: First, KSHV ORF45 inhibits activation of interferon regulatory factor 7 (IRF-7), and therefore antagonizes host innate antiviral response (28); Second, KSHV ORF45 interacts with p90 ribosomal kinase 1 and 2 (RSK1/RSK2) and modulates the ERK/RSK signaling pathway, which is known to play essential roles

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in KSHV reactivation and lytic replication (12). All these data suggest that KSHV ORF45 is a multifunctional protein.

ORF45 is unique to the gamma herpesviruses; it has no homologue in the alpha or beta herpesviruses. ORF45 homologues have been identified as virion protein components in other gamma herpesviruses, such as Epstein-Barr virus (EBV), rhesus rhadinovirus (RRV), and murine herpesvirus-68 (MHV-68), suggesting that certain tegument functions of ORF45 are conserved (2, 11, 18). ORF45 homologues differ in protein length. KSHV ORF45 is the longest, at 407 aa; RRV, EBV, MHV-68, and herpesvirus saimiri (HVS) have 353, 217, 206, and 257 aa, respectively. The limited homologies lie mostly at the ends of amino and carboxyl terminals. The middle portion of KSHV ORF45 diverges from those of its homologues. The homologues differ in subcellular localization. We and others have reported previously that KSHV ORF45 is found predominantly in the cytoplasm (1, 21, 28, 30), whereas ORF45 of MHV-68 is found exclusively in the nucleus (9). Recently we found KSHV ORF45 also present in the nucleus of BCBL-1 cells in what resembled viral replication compartments, suggesting that ORF45 could shuttle into the nucleus (12).

Nucleocytoplasmic trafficking of proteins across the nuclear membrane occurs through nuclear pore complexes. Small molecules of up to approximately 9 nm in diameter, corresponding to a globular protein of approximately 40-60 kDa, can in principle enter or leave the nucleus by diffusion through nuclear pores (15, 17, 24). Large molecules are transported with the aid of a related family of transport factors, importins and exportins, which recognize nuclear localization sequence (NLS)-containing or nuclear export sequence (NES)-containing proteins (15, 17, 23). CRM1 (exportin 1) has been identified as a common export receptor that recognizes HIV-REV like leucine-rich NES sequences and is responsible for the export of such NES-containing proteins (4, 5, 19, 22). The CRM1-dependent nuclear

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export is specifically inhibited by a pharmacological compound, leptomycin B (LMB), that interacts with CRM1 and thus blocks such NES-mediated protein export (4).

To understand the mechanism underlying the distinct intracellular distribution of KSHV ORF45, we attempted to locate the signals that control its subcellular localization. In the research reported here, we identified a leucine-rich NES and an adjacent basic NLS in KSHV ORF45. We demonstrated that the regulated intracellular trafficking of ORF45, especially its translocation into the nucleus, is important for KSHV lytic replication.

MATERIALS AND METHODS

Plasmids. We generated various EGFP-ORF45 fusion expression vectors by cloning PCR-amplified fragments in frames into pEGFP-C vector (Clontech). In each construct, the coding sequence of each of the ORF45s from different gamma herpesviruses was fused to the C-terminal of GFP. The template for amplifying KSHV ORF45 is pCR3.1-ORF45, which has been described previously (28). EBV ORF45 was amplified from cDNA of butyrate-induced BC-1 cells (27). The RRV ORF45 was amplified from a cosmid provided by Dr. Scott Wong, and HVS ORF45 was amplified from HVS viral DNA provided by Dr. Jae Jung. Similarly, PCR product of each ORF45 was also cloned into pKH3, a mammalian expression vector kindly provided by Dr. Ian Macara, to generate 3×HA-tagged ORF45 expression plasmids. Various KSHV ORF45 truncation mutants were also created by PCR amplification and cloning into pEGFP-C. Diagrams of these constructs are shown in Fig. 2A. The p2×EGFP-C2 vector with two tandem EGFPs was constructed by insertion of a Klenow-blunted *Ncol/BglII* EGFP fragment into the *Sma*I site of pEGFP-C2 and then removal of the extra A (adenosine) upstream of the *SalI* site by QuickChange mutagenesis (Stratagene, La Jolla, CA). Annealed oligonucleotides encoding the NES (NES-BglII, 5'-gatcgaagtattgagtcagagaatcgggctcatggacg-3' and NES-SalI, 5'-tcgacgtccatgagcccgattctctgactcaatacttc-3'), NES/NLS (NES/NLS-BglII, 5'-

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gatcgaagtattgagtcagagaatcgggctcatggacgtgggccagaagcgcaaaaggg-3' and NES/NLS-SalI, 5'-tcgacccttttgcgcttctggcccacgtccatgagcccgattctctgactcaatacttc-3'), or NLS (NLS-BglII, 5'-gatccagaagcgcaaaaggcagg-3' and NLS-SalI, 5'-tcgacctgccttttgcgcttctg-3') were inserted into p2×EGFP-C2 at the BglII and SalI sites to generate plasmids p2×EGFP-NES, p2×EGFP-NLS/NES, and p2×EGFP-NLS respectively. Plasmids p2×EGFP-284-383 and p2×EGFP-238-283 were generated by cloning the corresponding PCR fragments into p2×EGFP-C2. Site-directed mutagenesis of ORF45 was carried out with a QuickChange mutagenesis kit (Stratagene, La Jolla, CA). Primer sequences used for cloning and mutagenesis are available upon request. All clones were verified by DNA sequencing.

Cell Culture, transfection, and live fluorescent microscopy. 293T cells were cultured under 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics. Transient transfection of EGFP-fusion plasmids was performed in 24-well plate with Effectene transfection reagent (Qiagen, Valencia, CA). Twenty-four hours after transfection, cells expressing EGFP-fused proteins were observed under florescent microscope. In some cases, 10 ng/ml lepotomycin B (Sigma, St. Louis, MO) was added to the culture medium, and images were taken at the times indicated in Fig. 1, 2, and 3.

Indirect immunofluorescent staining. Cells cultured on cover slips in 24-well plate were transfected with 3×HA tagged ORF45 expression vectors. The transfected cells were fixed with 4% paraformaldehyde in PBS for 30min, permeabilized with 0.5% Triton X-100 for 5-10min, blocked with 2% BSA in PBS for 30 min, then incubated with primary antibody (1μg/ml) for 1 h. After three washes with PBS with 0.1% Triton X-100, the cells were incubated with Alexa-594 labeled secondary antibodies (Invitrogen, Carlsbad, CA) for 1h. The cells were also counterstained with DAPI (Sigma, St. Louis, MO) to detect nuclei. The cells were then mounted in anti-fade agent (Invitrogen, Carlsbad, CA) and visualized with a fluorescent microscope.

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Genetic manipulation of BAC36 with recombineering technology. The strategy for introducing point mutations into BAC36 by homogeneous recombination has been described previously (29). An *E. coli* strain, EL350, carrying BAC-del45, in which ORF45 coding sequence has been replaced with Kan/SacB double selection cassette, was used as starting material. The EL350 strain contains a defective λ prophage that harbors the recombination genes *exo, beta,* and *gam* under tight control of a temperature-sensitive cl857 repressor. Recombination functions can be transiently supplied by shifting of the culture to 42°C for 15 min (14). To generate a mutant with a desired mutation in ORF45 coding sequence, we replaced the Kan/SacB cassette of BAC-del45 with ORF45 coding sequence by homologous recombination. Two primers, a 78-bp ORF45wt5' (5'-tttccgcccctagcggtcaaccccgtacaaggccatggcgatgtttgtgaggacctcgtctagcacacacgatgaaga-3') and a 76-bp ORF45wt3'II (5'-gcatgagacttgacacctataatggtctgtattgacaccattcttttatttatcagtccagccacggccagttata-3'), were used to amplify the ORF45 coding region with plasmids carrying the desired mutations as templates. PCR was carried out at 94°C, 30 sec; 60°C, 30 sec; and 72°C, 2 min for 30 cycles with Expand High fidelity Taq polymerase (Roche, Indianapolis, IN). The PCR product was digested with *Dpn*I to remove the plasmid template. The digested product was gel-purified and electroporated into BAC36-del45–containing EL350 cells that had been induced at 42°C for 15 min. The parameters for electroporation were set at 1.75 KV, 276 ohms, and 50 mF in a 0.1-mm cuvette (BTX). After electroporation, all transformants were spreaded equally on ten 150 mm LB plates containing 12.5 μg/ml of chloramphenicol and 7% sucrose. The plates were incubated at 32°C overnight. Presumably as a result of repetitive sequences in viral genome, many of the Kan^S/$Sucrose^R$ colonies grew, but vast majority of them had unexpected recombinations. To identify the clones with desired recombination, we performed *in situ* colony hybridization to screen all colonies (about 50,000 or more) with wild-type ORF45 coding sequence as a probe. We usually obtained fewer than 10 colonies with positive hybridization signals.

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The positive clones were expanded, and the BAC DNAs were extracted and analyzed by PCR, restriction enzyme digestion, Southern blot, and sequencing analyses. The BAC DNAs with the proper recombination were prepared from overnight cultures with a Large-Construct kit (Qiagen, Valencia, CA).

Reconstitution of recombinant KSHV viruses. Briefly, 293T cells seeded in a 60-mm dish were transfected with 2 µg of BAC DNAs with Lipofectamin 2000 (Invitrogen, Carlsbad, CA). Two days after transfection, cells were subcultured into a T150 flask with fresh medium containing 200 µg/ml hygromycin. After 8 days of selection, hygromycin-resistant colonies were trypsinized, pooled, and subcultured with 1:3 dilution every 3 days. To induce viral lytic replication, we seeded 3×10^7 BAC-containing 293T cells in T150 flask, and 2 days later, we replaced the medium with fresh one containing 20 ng/ml TPA and 0.3 mM butyrate.

Virus stock preparation and infection. Usually, six or more T-150 flasks of cells were induced for 4 to 5 days, and viruses were concentrated from the supernatant. The induced medium was collected and centrifuged to remove cell debris. The cleared supernatant was filtered through a 0.45 µm filter, and virions were pelleted at 100,000 g for 1 h on a 25% sucrose cushion with a Beckman SW28 rotor. The virus pellets were dissolved in 1% of original volume of phosphate-buffered saline (PBS) or DMEM and stored at –80°C. Viral genome copy number of stock virus was then quantified by real-time quantitative PCR. Infection was carried out as previously described (29). Briefly, 293T cells plated in 24-well plates were incubated with concentrated virus plus polybrene (4 µg/ml) and spun at 800 g for 1 h at room temperature. The plates were then incubated at 37°C for another 2 h and the inocula were then removed and replaced with fresh media with 5% FBS. Next day, the media were replaced with fresh media containing 1% FBS. The plates were examined with an inverted fluorescence microscope 48 h after infection for cells expressing GFP.

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Real-time quantitative PCR analysis of virion DNA. After TPA/butyrate induction, medium from induced BAC-293T cells was collected, centrifuged, and passed through a 0.45 µm filter to clear cell debris. Treatment of 200 µl of the cleared supernatant with 10 units of Turbo DNase (Ambion, Austin, TX) at 37°C for 1 h degraded extra-virion DNAs. The reaction was stopped by addition of EDTA followed by heat inactivation at 70°C. Then 20 µl of proteinase K solution and 200 µl of Buffer AL from the DNeasy Kit (Qiagen, Valencia, CA) were added. The mixture was kept at 70°C for 15 min and then extracted with phenol/chloroform. The DNA was ethanol precipitated with glycogen as a carrier, and the DNA pellet was dissolved in 40 µl of TE buffer. Two microliters of such DNA was used in a real-time quantitative PCR reaction with the LightCycler FastStart DNA Master^{Plus} SYBR Green Kit as we described previously (29). Viral DNA copy numbers were calculated with external standards of known concentrations of BAC36 DNA.

Western blotting. Cells were washed with PBS and lysed with whole cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate [Na_3VO_4], 40 mM β-glycerophosphate, 30 mM sodium fluoride, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml of aprotinin, 5 µg/ml of leupeptin, 5 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride) to produce whole cell lysates for most western blot analyses. In the western blot experiments described in Fig. 4C, the cytoplasmic and nuclear extracts were used instead. Briefly, HEK293T cells reconstituted with BAC36 or mutant BACs were induced with TPA and sodium butyrate together for 3 days. Cells were washed with cold PBS and then resuspended in the hypotonic lysis buffer (10mM HEPES, pH 7.9, 1.5mM $MgCl_2$, 10mM KCL, 0.5mM DTT, protease inhibitor cocktail), and incubated on ice with gently mixing from time to time for 15 min. The resulting suspensions were centrifuged at 1500 g for 15 min and the supernatants were collected as cytoplasmic extracts. The pellets were washed once with the hypotonic lysis buffer, and then resuspened in whole

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cell lysis buffer, and cleared by high speed centrifugation to produce nuclear extracts. The protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce Biotech., Rockford, IL). About 50 micrograms of cell extracts of each sample were resolved on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% dried milk in PBS plus 0.2% Tween 20 (PBST) and then incubated with diluted primary antibodies for 2 h at room temperature or 4°C overnight. Anti-rabbit or anti-mouse IgG antibodies conjugated to horseradish peroxidase (Pierce Biotech., Rockford, IL) were used as the secondary antibodies. Supersignal chemiluminescence system (Pierce Biotech., Rockford, IL) was used for detection of antibody-antigen complex. Mouse antibodies against KSHV ORF45, K8, and ORF21 have been described before (26). Mouse monoclonal anti-RTA and anti-ORF65 were gifts from K. Ueda and S.-J. Gao respectively. Rat anti-LANA antibody was purchased from Advanced Biotechnologies, Inc. (Columbia, MD). Mouse monoclonal anti-EGFP was purchased from Clontech Inc. (Mountain View, CA). Anti-HA and anti-β-actin were purchased from Sigma (St. Louis, MO).

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RESULTS

Difference in subcellular localization of KSHV ORF45 from its homologues in other gamma herpesviruses. We and others have observed that KSHV ORF45 protein is localized primarily in the cytoplasm (1, 21, 28, 30) and (Fig. 1A), whereas its homologue MHV-68 ORF45 is mostly seen in the nucleus (9). To examine the subcellular localization of ORF45s encoded by other gamma herpesviruses, we tagged each ORF45 with EGFP and examined the fusion protein in living transfected 293T cells with fluorescent microscopy. Both RRV and HVS ORF45s were seen exclusively in the nucleus, whereas EBV ORF45 homolog BKRF4 was present in both cytoplasm and nucleus (Fig. 1A). Fusion of EGFP to either N- or C- terminal of KSHV ORF45 resulted in the same subcellular localization of the native protein revealed by immunofluorescent assay (IFA) with specific anti-KSHV ORF45 antibodies (28, 30). Because antibodies to other ORF45s were not available, each ORF45 from different gamma herpesviruses was tagged with HA and its subcellular localization was examined by IFA with anti-HA antibody. In all cases, HA-tagged and EGFP-tagged proteins showed identical localizations, suggesting that the distinct subcellular localization is intrinsic characteristics of the ORF45 protein itself and is not affected by EGFP or HA tags (Fig. 1B).

Recently, we observed that KSHV ORF45 in the induced BCBL-1 cells was localized predominantly in the cytoplasm but a portion accumulated in the nucleus (12). We therefore speculated that KSHV ORF45 could shuttle between nucleus and cytoplasm. To determine whether it can, we treated EGFP-ORF45 transfected cells with LMB, which specifically inhibits CRM1-dependent nuclear export. We found that KSHV ORF45 accumulated rapidly in the nucleus after LMB treatment (Fig. 1C). This result indicates that KSHV ORF45 could shuttle between cytoplasm and nucleus and possibly contains a CRM1-dependent NES signal.

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Control of subcellular localization by signals located between amino acids 284 and 310 of KSHV ORF45. To identify the sequence elements that control KSHV ORF45 subcellular localization, we constructed a series of EGFP-fused ORF45 truncation mutants (Fig. 2A). Western blot analyses revealed that all constructs are expressed and largely in lines with their expected sizes (Fig. 2B). Progressive truncations up to aa 310 from the carboxyl terminal have no effect on the cellular localization of EGFP-ORF45 fusion proteins. All these EGFP-fusion mutants including aa 1–383, 1–331, and 1–310 were predominantly cytoplasmic in untreated cells. After LMB treatment, these truncated EGFP-ORF45 fusion mutants accumulated in the nucleus (Fig. 2A and 2C), indicating that the putative NES element was localized to the N-terminal 310 aa. Truncation from aa 310 to 294 did not affect the cytoplasmic localization, suggesting the NES remains intact, but the nuclear accumulation of the remaining aa 1–294 became less sensitive to LMB treatment (Fig. 2A and 2C). Further truncation from aa 294 to 284 drastically changed the subcellular localization, and the resultant aa 1–283 was not restricted to the cytoplasm but evenly distributed in both cytoplasm and nucleus, like EGFP itself. Smaller truncation mutants aa 1–237 and 1–115 displayed the similar pattern as aa 1–283. These results suggest that the region between aa 284 and 294 confers ORF45 cytoplasmic localization and possibly contains an NES. The region between aa 294 and 310 seems to increase nuclear localization, and deletion of this region results in less efficient nuclear accumulation. The results could be explained by existence of an NLS in this region. Results from N-terminal progressive truncations support this interpretation. Deletion up to 238 aa did not affect ORF45's predominant cytoplasmic localization. EGFP-ORF45 fusion mutants aa 116-383 and 238–383 were both mostly found in the cytoplasm and highly sensitive to LMB treatment, so they may both contain functional NES and NLS. The mutant aa 284–383 was seen in both the cytoplasm and the nucleus even though it contains a putative NES, presumably because its small size enables it to pass through nuclear pores. Indeed, when it was fused

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with 2 tandem EGFP, the fusion protein became localized predominantly in the cytoplasm (see below in Fig. 3C). Although other possibilities might exist, for an example, the neighboring sequence could regulate the NES activity, its high sensitivity to LMB suggests the aa 284–383 region preserves a functional NES. Other mutants that do not have the region between aa 284 and 310, including mutants aa 311–383 and 332–407 show diffusive patterns like that of EGFP and are not sensitive to LMB treatment. In conclusion, the region aa 284–310 of KSHV ORF45 contains important signals that control its subcellular localization.

Identification of a leucine-rich NES and an adjacent NLS in KSHV ORF45. Inspection of the sequence in the region revealed that residues ^{284}VLSQRIGLMDV^{294} contain a stretch of hydrophobic amino acids resembling an NES consensus (ØX$_{2-3}$ØX$_2$ØXØ) (Fig. 3A, Ø represents any hydrophobic amino acid, like leucine, isoleucine, valine, tryptophan or methonine, whereas X represents any amino acid) (13, 23), and the region aa 297–300 contains a typical basic-residue-rich NLS signal, ^{297}KRKR^{300} (16). To determine whether residues aa 284–294 are a functional NES, we introduced point mutations into this region to replace the conserved hydrophobic residues with alanines. Replacement of valine 284 and leucine 285 with alanines within the context of full-length ORF45 leads to nuclear accumulation of the mutated EGFP-ORF45 (V284A/L285A) fusion proteins (Fig. 3B, panel C). Similarly, changing isoleucine 289 and leucine 291 to alanines had a similar result, and the resultant EGFP-ORF45 (I289A/L291A) was exclusively seen in the nucleus (Fig. 3B, panel D). These results indicate that these hydrophobic residues are important for nuclear export of ORF45. These two mutants are designated ORF45-rn1 (restricted to the nucleus) and ORF45-rn2. To determine whether the adjacent basic residues ^{297}KRKR^{300} are a functional NLS, we replaced the basic residues with alanines. We found that the resulting fusion protein was restricted to the cytoplasm, and diffusely distributed in

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both cytoplasm and nucleus even after LMB treatment (Fig. 3B, bottom panels). This mutant is designated ORF45-rc (restricted to the cytoplasm).

To determine whether ^{297}KRKR^{300} and ^{284}VLSQRIGLMDV^{294} function as authentic NLS and NES respectively, we fused corresponding coding sequences in frame between two EGFPs and examined the subcellular localization of the fusion protein. The 2×EGFP alone is present in both nucleus and cytoplasm (Fig. 3C, panel A). The addition of NLS ^{297}KRKR^{300} to the 2×EGFP resulted in a 2×EGFP-NLS fusion protein that is exclusively nuclear (Fig. 3C, panel B), suggesting ^{297}KRKR^{300} is a functional NLS. Fusion of the NES to the 2×EGFP resulted in predominantly cytoplasmic 2×EGFP-NES fusion protein, suggesting ^{284}VLSQRIGLMDV^{294} is a functional NES (Fig. 3C, panel D). Inclusion of both NES and NLS resulted in a mostly cytoplasmic 2×EGFP-NES/NLS fusion proteins that rapidly accumulated into nuclei after LMB treatment, suggesting ^{284}VLSQRIGLMDV^{294} has authentic NES activity that counteracts the NLS activity (Fig. 3C, panel C). Fusion of KSHV ORF45 aa 284-383 which contains the NES/NLS signals to the 2×EGFP gave similar results (Fig. 3C, compare panels E and C). However, insertion of aa 238-283 which lacks the NES or NLS signal into the 2×EGFP resulted in a pancellular fusion protein not responsive to LMB treatment (Fig. 3C, panel F).

Functional roles of ORF45 NES and NLS in the KSHV lytic life cycle. Next, we examined the effect of alteration of ORF45 subcellular localization on KSHV lytic replication. These mutations were introduced into the KSHV viral genome BAC36 by recombineering as previously described (25, 29). The clones with expected recombination were selected on a Kan^S/Sucrose^R plate, then subjected to *in situ* hybridization and further analyzed by restriction-enzyme analysis (Fig. 4A). All these mutants showed Kpn I digestion patterns similar to that of wild-type BAC36 which generated two fragments of 3.7 kb and 2.2 kb, but are distinct from that of parental BAC-del45 which yielded two up-shifted bands of 4.3 kb and 3.4 kb (Fig. 4A), so proper recombination is at the expected locus. Direct sequencing of

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each clone also confirmed expected mutations (data not shown). These BAC DNAs were transfected into 293T cells, which were then subjected to hygyromycin selection. The pooled hygromycin-resistant and GFP-positive cells were induced with TPA/butyrate, which initiated lytic viral replication. Whole cell lysates were prepared and analyzed for expressions of several viral proteins. Except ORF45-null BAC-stop45, which do not express ORF45 as expected, other ORF45 NES or NLS mutants express comparable levels of viral proteins including ORF45, RTA, ORF21, ORF65, and LANA as the wild type BAC36-wt (Fig. 4B). We next examined whether the mutant viruses had the expected ORF45 subcellular localizations. Because the KSHV BAC-reconstituted 293T cells adhere very poorly to culture surface, we had difficulty to perform immunofluorescence staining to visualize the subcellular localization of ORF45. Instead, we isolated cytoplasmic and nuclear extracts of the induced cells and analyzed them by western blot. As shown in the figure 4C, a stronger signal was detected in the cytoplasmic fraction of wild type BAC36-293T cells than in the nuclear one, confirming the mainly cytoplasmic localization of KSHV ORF45 (Fig. 4C, compare lanes 2 to 1). Also as expected, ORF45 was not expressed in the BAC-stop45-293T cells (Fig. 4C, lanes 3 and 4). ORF45 was equally detected in both cytoplasmic and nuclear fractions of cells reconstituted with BAC-45rn1 (Fig. 4C, compare lanes 5 and 6) or BAC-45rn2 (data not shown), suggesting that the BAC-45rn mutants became more nuclear than the wild type because of loss of functional NES. In contrast, the signal of ORF45 was much stronger in the cytoplasmic extract than in the nuclear one of cells reconstituted with BAC-45rc, in which the NLS has been destroyed, confirming its more cytoplasmic phenotype. These results largely confirmed the expected subcellular localizations of the wild type ORF45 and the mutants. Interestingly, the nuclear and cytoplasmic ORF45 proteins apparently differ in mobility on SDS-PAGE gel, suggesting they are post-translationally modified differently. The difference on the electrophoretic mobility is at least partially caused by phosphorylation (unpublished data). Having confirmed the expression and

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subcellular localization of each ORF45 mutant in the reconsitituted cells, we next examined the growth curve of each virus. Extracellular viral particles were collected from induced cells, and the viral genome copies were determined by real time PCR. A single-step growth curve showed that BAC-45rn1 and BAC-45rn2 mutants, in which NES is nonfunctional and ORF45 protein is therefore restricted to the nucleus, had a growth curve similar to that of the BAC36-wt. In contrast, BAC-45rc mutant, in which the NLS has been destroyed, appeared to have a growth defect like that of the ORF45-null BAC-stop45 (Fig. 4D). The viruses collected 4 days after induction were analyzed by western blot for virion proteins ORF45 (tegument), ORF21 (tegument), and ORF65 (capsid), and significantly less virion protein was found in the BAC-stop45 and BAC-45rc preparations (Fig. 4E), confirming lower yields of these two mutants. The viruses collected were also used to infect 293T cells, and infectivity was judged by the appearance of green fluorescent cells upon inoculation of fresh 293T cells. In agreement with the titration by real-time PCR and western blot of virion proteins, we found that BAC-45rn1 and BAC-45rn2 produced numbers of infectious viruses similar to that of the wild-type BAC36. However, we found that BAC-45rc as well as ORF45-null BAC-stop45 produced significantly fewer infectious viruses (Fig. 4F). These results suggested that the NES and NLS have functional roles in KSHV lytic replication.

DISCUSSION

The difference between the subcellular localizations of KSHV ORF45 (mainly cytoplasmic) and MHV68 ORF45 (mainly nuclear) prompted us to examine ORF45s of other gamma herpesviruses. We tagged EGFP or HA with several ORF45 homologues and determined the subcellular localizations of the EGFP-fusion proteins in 293T cells. We found only KSHV ORF45 to be exclusively cytoplasmic; its homologues from other gamma herpesviruses were either exclusively nuclear (RRV and HVS) or evenly distributed in both cytoplasm and nucleus (EBV). We recently observed a portion of KSHV ORF45 in

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the nucleus in BCBL-1 cells (12). Presence in the nucleus therefore seems to be common for ORF45s of gamma herpesviruses. In order to understand the mechanism of distinct subcellular localization of KSHV ORF45, we performed detailed mutational analysis to identify the signals that control its subcellular localization. Analysis of the progressive truncation mutants revealed that the region between aa 284 and 310 is critical for KSHV ORF45 subcellular localization. Examination of the sequence revealed a leucine-rich-like NES (^{284}VLSQRIGLMDV^{294}) and adjacent NLS (^{297}KRKR^{300}) in this region. The exclusive cytoplasmic localization of KSHV ORF45 is mainly mediated by this CRM1-dependent NES. Treatment with CRM1-specific inhibitor LMB or replacement of the NES consensus hydrophobic amino acids with alanines disrupts KSHV ORF45 nuclear export and results in its rapid nuclear accumulation. This NES is transferable because it can direct nuclear export of 2×GFP (Fig 3C, compare panel D to A). The adjacent sequence (^{297}KRKR^{300}) seems to be an authentic NLS signal. It is also transferable and is sufficient to convert 2×GFPs from pancellular to completely nuclear localization (Fig. 3C, compare panel B to A). This NLS is important for nucleus/cytoplasm shuttling of ORF45 because replacement of the basic residues with alanines resulted in drastically reduced efficiency of nuclear accumulation in the presence of LMB (Fig. 3B, panels E and F).

The common nuclear presence of KSHV ORF45 and its homologues suggests a conserved function in the nucleus. This nuclear function seems to be important for viral replication, possibly at the late stage, because both BAC-45rn1 and BAC-45rn2 have growth curves similar to that of BAC36-wt, but BAC-45rc grows similarly to the ORF45-null BAC-stop45. Previous studies have shown KSHV ORF45 has important functions at both early and late stages of viral replication, but its exact roles remained to be elucidated (29). Among all other gamma herpesviruses, only MHV68 ORF45 has been characterized (9, 10). By SiRNA-based ablation of gene expression or BAC-based mutagenesis, Jia et al. (9, 10) demonstrated that MHV68 ORF45 is essential for MHV68 replication, but again the exact

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function was not demonstrated. Interestingly, the authors showed that ectopic expression of an NLS deletion mutant of ORF45 could partially rescue ORF45-null MHV-68, suggesting that the NLS is not absolutely essential for MHV68 ORF45. This result seems to contradict our observation that a functional KSHV ORF45 NLS is important for viral replication. Because MHV68 ORF45 protein is so small (206 aa), it could possibly cross the nuclear pore complexes by diffusion even in the absence of a functional NLS. Importantly, Jia et al. (9) also demonstrated that KSHV ORF45 could partially rescue an MHV-68 ORF45-deficient mutant, suggesting a certain nuclear function is conserved among gamma herpesviruses.

What is the possible function of KSHV ORF45 in the nucleus? It is a relatively abundant tegument protein located between the capsid and virion envelope in the virion particle. Early evidence suggests that KSHV ORF45 is located in the inner layer of tegument and is tightly associated with capsid (26, 30). A recent virion-wide interaction study determined that ORF45 interacts with capsid protein ORF62, a triplex component (20). Genetic analysis revealed that ORF45 could have a role in the late stage of virion maturation that takes place after viral DNA replication (29). In the BCBL-1 cells, a portion of ORF45 is found to be colocalized with the viral replication compartment where viral DNA replication and initial capsid assembly occurs (12). These observations suggest that ORF45 could be one of the tegument proteins that are acquired in the nucleus soon after capsid assembly. The association of ORF45 with the capsid is probably important to subsequent virus maturation processes. Further studies are needed that clarify role of ORF45 in this late stage of viral replication.

In addition to its viral functions as a tegument component, KSHV ORF45 interacts with a number of cellular proteins and has important roles in modulation of the host cellular environment. Two cellular functions have been reported for KSHV ORF45: (1) inhibition of IRF-7 activation and evasion of host antiviral response (28), and (2) activation of RSK1/RSK2 and modulation of the ERK MAPK

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signaling pathway (12). Our preliminary data suggested that alternation of the subcellular localization of KSHV ORF45 has little effect on its binding to IRF-7 or to RSK1/RSK2. All NES and NLS mutants are still able to inhibit IRF-7 activation in a luciferase reporter assay (data not shown). These mutants retain their abilities to activate RSK1 and RSK2 when they are overexpressed in the cells (data not shown), but change of subcellular localization may result in a difference in local concentration of ORF45 that could have a dramatic functional consequence and may not be revealed by the gross activity assays.

KSHV ORF45 is the only one in the gamma herpesviruses that appears exclusively in the cytoplasm, suggesting that a relatively strong NES signal exists in the KSHV ORF45 sequence. Mutation of the key hydrophobic residues in KSHV ORF45 NES drastically changes its predominantly cytoplasmic to a nuclear localization. Fusion of KSHV ORF45 NES/NLS or NES to 2×GFP result in an exclusive cytoplasmic localization (Fig. 3C), further confirming the ^{284}VLSQRIGLMDV^{294} sequence possesses authentic and transferable NES activity. Leucine-rich NES is known to be subject to various regulations (13). The mechanism by which subcellular localization is regulated and whether it is regulated over the course of KSHV infection will be interesting to determine. Upon KSHV infection of human fibroblast cells followed by RTA-adenovirus infection, ORF45 was seen throughout the cell at 8h pi, then predominantly nuclear at 24h, suggesting that ORF45 relocates over the course of infection (Glaunsinger, personal communication). Phosphorylation of residues in the vicinity of the NES and NLS may regulate the intracellular distribution of a protein (8, 13). ORF45 is rich in serine and threonine residues and is heavily phosphorylated in the cells (30). The slower electrophoretic mobility of the cytoplasmic fraction suggests that ORF45 is post-translationally modified (including possible phosphorylation) more extensively in the cytoplasm than in the nucleus. Whether the subcellular localization of ORF45 is regulated by phosphorylation or other modifications remains to be determined.

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The only known kinases that phosphorylate ORF45 are RSK1 and RSK2, but the major phosphorylation sites of RSKs lie in the region aa 1–115, which is dispensable for the cytoplasmic-dominant localization (12), so that RSKs are unlikely to be directly involved in the regulation of ORF45 subcellular localization.

KSHV ORF45 is larger in size than its homologues. The middle portion of the KSHV ORF45 amino acid sequence, including the NES/NLS and the possible regulatory region, seems to be "extra" and has no homology to other gamma herpesviruses. The putative basic-residue-rich NLS can be found at different positions in ORF45s of all gamma herpesviruses, suggesting that a common nuclear function is conserved and possibly required for efficient viral lytic replication and progeny virus production. Although the NES seems dispensable for KSHV lytic replication, it might be important for certain ancillary functions gained by KSHV during evolution that could facilitate its interaction with the host. The different modifications on nuclear and cytoplamic fractions further support that ORF45 has distinct functions in the nucleus and the cytoplasm. Nevertheless, identification of the subcellular localization signals of ORF45 opens an avenue to further characterize the functions of this protein in KSHV life cycle.

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REFERENCES

1. **Abada, R., T. Dreyfuss-Grossman, Y. Herman-Bachinsky, H. Geva, S. R. Masa, and R. Sarid.** 2008. SIAH-1 interacts with the Kaposi's sarcoma-associated herpesvirus-encoded ORF45 protein and promotes its ubiquitylation and proteasomal degradation. J Virol **82:**2230-2240.
2. **Bortz, E., J. P. Whitelegge, Q. Jia, Z. H. Zhou, J. P. Stewart, T. T. Wu, and R. Sun.** 2003. Identification of proteins associated with murine gammaherpesvirus 68 virions. J Virol **77:**13425-13432.
3. **Dourmishev, L. A., A. L. Dourmishev, D. Palmeri, R. A. Schwartz, and D. M. Lukac.** 2003. Molecular genetics of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) epidemiology and pathogenesis. Microbiol Mol Biol Rev **67:**175-212.
4. **Fornerod, M., M. Ohno, M. Yoshida, and I. W. Mattaj.** 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell **90:**1051-1060.
5. **Fukuda, M., S. Asano, T. Nakamura, M. Adachi, M. Yoshida, M. Yanagida, and E. Nishida.** 1997. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature **390:**308-311.
6. **Ganem, D.** 2007. Kaposi's sarcoma-associated herpesvirus, p. 2847-2888. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields Virology, 5 ed, vol. 2. Lippincott Williams & Wilkins, Philadelphia, PA.
7. **Ganem, D.** 2006. KSHV infection and the pathogenesis of Kaposi's sarcoma. Annu Rev Pathol **1:**273-296.
8. **Jans, D. A., and S. Hubner.** 1996. Regulation of protein transport to the nucleus: central role of phosphorylation. Physiol Rev **76:**651-685.
9. **Jia, Q., V. Chernishof, E. Bortz, I. McHardy, T. T. Wu, H. I. Liao, and R. Sun.** 2005. Murine gammaherpesvirus 68 open reading frame 45 plays an essential role during the immediate-early phase of viral replication. J Virol **79:**5129-5141.
10. **Jia, Q., T. T. Wu, H. I. Liao, V. Chernishof, and R. Sun.** 2004. Murine gammaherpesvirus 68 open reading frame 31 is required for viral replication. J Virol **78:**6610-6620.
11. **Johannsen, E., M. Luftig, M. R. Chase, S. Weicksel, E. Cahir-McFarland, D. Illanes, D. Sarracino, and E. Kieff.** 2004. Proteins of purified Epstein-Barr virus. Proc Natl Acad Sci U S A **101:**16286-16291.

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12. **Kuang, E., Q. Tang, G. G. Maul, and F. Zhu.** 2008. Activation of p90 ribosomal S6 kinase by ORF45 of Kaposi's sarcoma-associated herpesvirus and its role in viral lytic replication. J Virol **82:**1838-1850.
13. **Kutay, U., and S. Guttinger.** 2005. Leucine-rich nuclear-export signals: born to be weak. Trends Cell Biol **15:**121-124.
14. **Lee, E. C., D. Yu, J. Martinez de Velasco, L. Tessarollo, D. A. Swing, D. L. Court, N. A. Jenkins, and N. G. Copeland.** 2001. A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics **73:**56-65.
15. **Mattaj, I. W., and L. Englmeier.** 1998. Nucleocytoplasmic transport: the soluble phase. Annu Rev Biochem **67:**265-306.
16. **Nakai, K., and P. Horton.** 1999. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem Sci **24:**34-36.
17. **Nigg, E. A.** 1997. Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature **386:**779-787.
18. **O'Connor, C. M., and D. H. Kedes.** 2006. Mass spectrometric analyses of purified rhesus monkey rhadinovirus reveal 33 virion-associated proteins. J Virol **80:**1574-1583.
19. **Ossareh-Nazari, B., F. Bachelerie, and C. Dargemont.** 1997. Evidence for a role of CRM1 in signal-mediated nuclear protein export. Science **278:**141-144.
20. **Rozen, R., N. Sathish, Y. Li, and Y. Yuan.** 2008. Virion-wide protein interactions of Kaposi's sarcoma-associated herpesvirus. J Virol **82:**4742-4750.
21. **Sander, G., A. Konrad, M. Thurau, E. Wies, R. Leubert, E. Kremmer, H. Dinkel, T. Schulz, F. Neipel, and M. Sturzl.** 2008. Intracellular localization map of human herpesvirus 8 proteins. J Virol **82:**1908-1922.
22. **Stade, K., C. S. Ford, C. Guthrie, and K. Weis.** 1997. Exportin 1 (Crm1p) is an essential nuclear export factor. Cell **90:**1041-1050.
23. **Tran, E. J., T. A. Bolger, and S. R. Wente.** 2007. SnapShot: nuclear transport. Cell **131:**420.
24. **Wang, R., and M. G. Brattain.** 2007. The maximal size of protein to diffuse through the nuclear pore is larger than 60kDa. FEBS Lett **581:**3164-3170.

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25. **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:**6185-6196.
26. **Zhu, F. X., J. M. Chong, L. Wu, and Y. Yuan.** 2005. Virion proteins of Kaposi's sarcoma-associated herpesvirus. J Virol **79:**800-811.
27. **Zhu, F. X., T. Cusano, and Y. Yuan.** 1999. Identification of the immediate-early transcripts of Kaposi's sarcoma-associated herpesvirus. J Virol **73:**5556-5567.
28. **Zhu, F. X., S. M. King, E. J. Smith, D. E. Levy, and Y. Yuan.** 2002. A Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF-7 phosphorylation and nuclear accumulation. Proc Natl Acad Sci U S A **99:**5573-5578.
29. **Zhu, F. X., X. Li, F. Zhou, S. J. Gao, and Y. Yuan.** 2006. Functional characterization of Kaposi's sarcoma-associated herpesvirus ORF45 by bacterial artificial chromosome-based mutagenesis. J Virol **80:**12187-12196.
30. **Zhu, F. X., and Y. Yuan.** 2003. The ORF45 protein of Kaposi's sarcoma-associated herpesvirus is associated with purified virions. J Virol **77:**4221-4230.

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FIGURE LEGENDS

FIG. 1. Different subcellular localization of KSHV ORF45 than of its homologues in other gamma herpesviruses. (A) Subcellular localizations of KSHV ORF45 and its homologues. EGFP-fused ORF45s expression vectors were transfected into 293T cells. Green fluorescence was visualized 24 h after transfection. As indicated, some transfected cells were treated with 10 ng/ml leptomycin B (LMB) which specifically inhibits CRM1-dependent nuclear export. (B) HA-tagged ORF45 expression vectors were transfected into 293T cells. The transfected cells were fixed, permeabilized, and immunofluorescently stained with anti-HA antibodies (red). The cells were also counterstained with DAPI to show the nucleus (blue). (C) KSHV ORF45 rapidly accumulates in the nucleus after LMB treatment. EGFP-fused KSHV ORF45 was visualized at different times, as indicated, after LMB treatment.

FIG. 2. Mapping of the subcellular localization signals of KSHV ORF45. (A) Schematic representation of subcellular localizations of KSHV ORF45 full-length and truncation mutants. HEK 293T cells were transfected with EGFP-ORF45 fusion constructs and treated with LMB for 30 min at 24 h after transfection. GFP was then visualized with fluorescent microscopy. Subcellular localization and LMB sensitivity of EGFP-ORF45 mutants are summarized on the right: C, predominantly cytoplasmic; N, predominantly nuclear; ++, translocates into nucleus within 30 min of LMB treatment; +, remains evenly distributed in both cytoplasm and nucleus after 120 min of LMB treatment. (B) Western blot of the full-length and truncated EGFP-ORF45 fusion proteins. Cell lysates of HEK 293T cells transfected with EGFP-ORF45 fusion constructs were analyzed by western blot with anti-GFP antibody. The sizes of molecular weight standards are shown on the right. (C) Representative images of full-length and truncated EGFP-ORF45 fusion proteins expressed in 293T cells in the presence (+LMB) and absence of (-LMB) of leptomycin B treatment.

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FIG. 3. Identification of functional nuclear export signal (NES) and adjacent nuclear localization signal (NLS) of KSHV ORF45. (A) Comparison of the putative ORF45 NES with other leucine-rich HIV-1 REV-like NESs (13). Conserved hydrophobic residues (primarily leucines) are shown in red. In the NES consensus sequence, X is any amino acid and Ø is any hydrophobic residue as indicated. A schematic representation of ORF45 bipartite NES/NLS motif is shown at the bottom with key amino acids highlighted. (B) Subcellular localization of EGFP-ORF45 wild type and mutants with altered NES or NLS sequences. The responses to LMB are also shown as indicated for ORF45-WT and for ORF45-rc whose NLS has been mutated. (C) Subcellular localization of 2×EGFP fused with KSHV ORF45 NLS and/or NES. The KSHV-ORF45 NLS and NES sequences were inserted in frame between two EGFPs. The constructs were transfected into HEK 293T cells. Twenty-four hours after transfection, the cells are treated with LMB for 30 min. GFP was then visualized with fluorescent microscopy. Representative images and schematic illustration of these constructs are shown.

FIG. 4. Role of ORF45 nuclear export and localization signals in KSHV replication. (A) Recombinant KSHV BACs with mutated NES or NLS sequence in ORF45. The wild type BAC36 and mutant KSHV BAC DNAs were constructed and prepared as described in Materials and Methods. The BAC DNAs were digested with *Kpn*I, resolved on an 0.8% agarose gel, and stained with ethidium bromide. The arrows on the left mark the differences between NES/NLS mutants and their parental BAC-del45, in which ORF45 coding sequence is replaced with a double selection marker Kan/SacB. The sizes of molecular makers are shown on the right. (B) Expression of viral proteins. Recombinant viruses were reconstituted in 293T cells, and lytic replications were induced by TPA and sodium butyrate. Cell lysates were analyzed by western blot with specific antibodies against KSHV viral proteins. The blot was also probed with anti-β-actin for loading control. (C) Subcellular localization of ORF45 in viral infected cells. HEK293T cells reconstituted with wild type BAC36 or mutant BACs

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were induced with TPA and sodium butyrate for 3 d. The nuclear and cytoplasmic extracts were prepared and analyzed by western blot to detect ORF45. The blot was also probed to detect lamin A and β-tubulin as nuclear and cytoplasmic fraction markers respectively. (D) Growth curves of recombinant KSHV BAC viruses. Supernatants of induced medium were collected at different time points as indicated. Viral genome copies in the medium were determined by real-time PCR and were plotted over time since induction. (E) Western blot analysis of virion lysates. The virions secreted in the medium 4 days after induction were collected, purified, and analyzed by western blot for virion proteins ORF45, ORF21 (tegument), and ORF65 (capsid). (F) Infection of 293T cells by the reconstituted recombinant viruses. Concentrated extracellular viruses were used to infect 293T cells. The infected cells express GFP and appear green when examined under fluorescent microscopy.

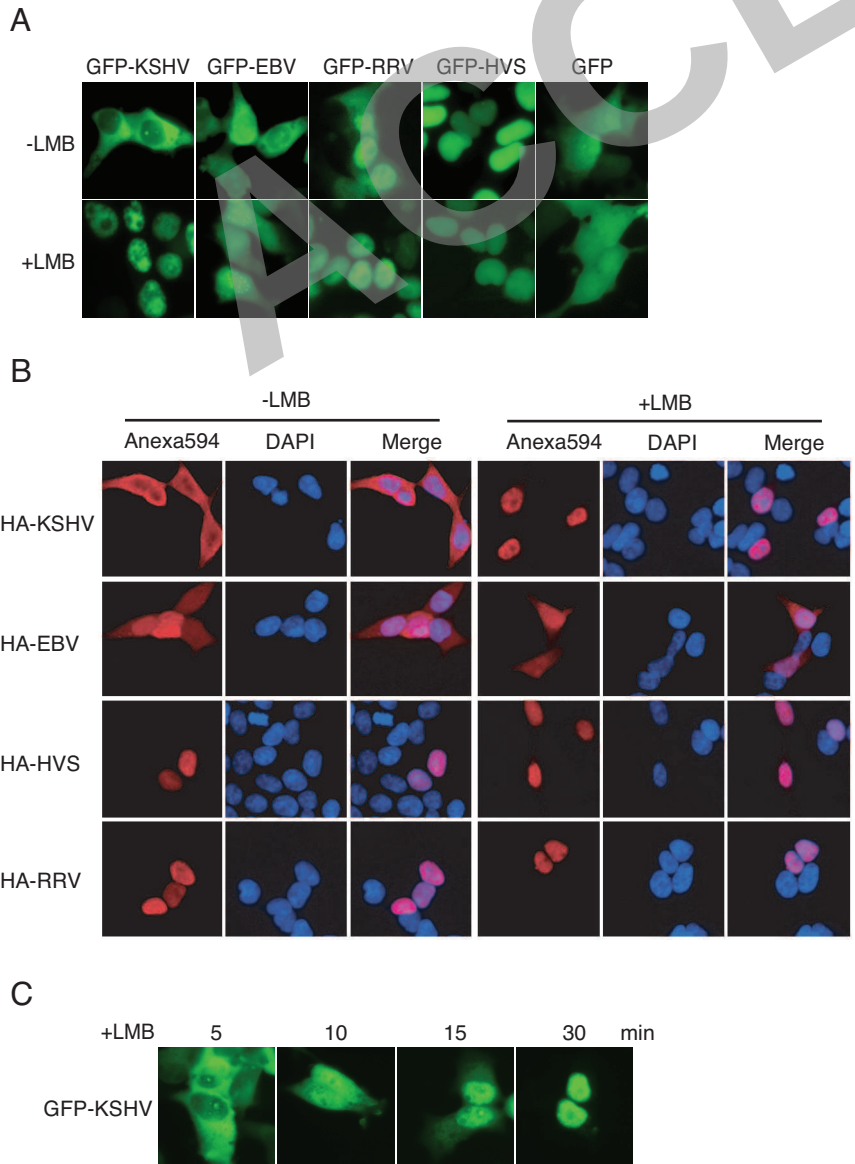
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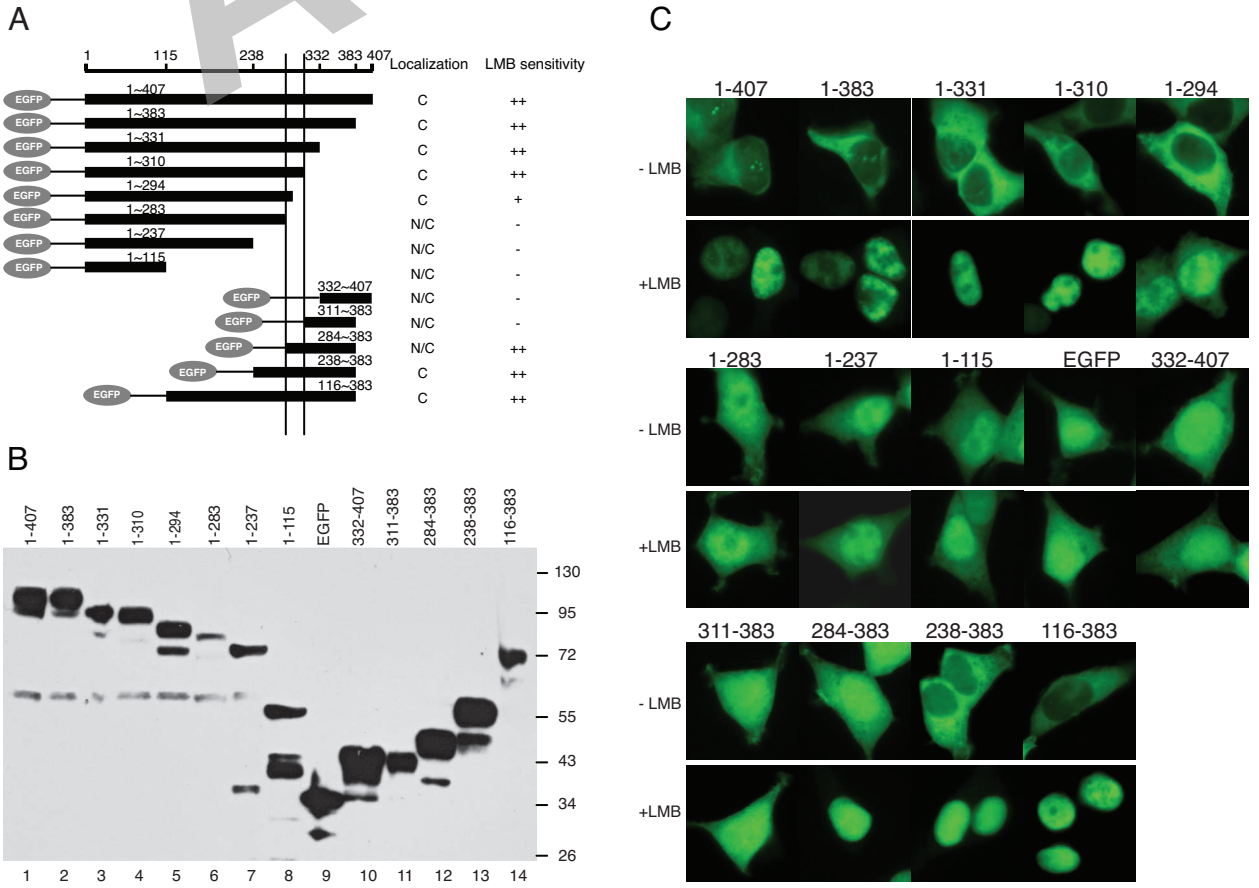
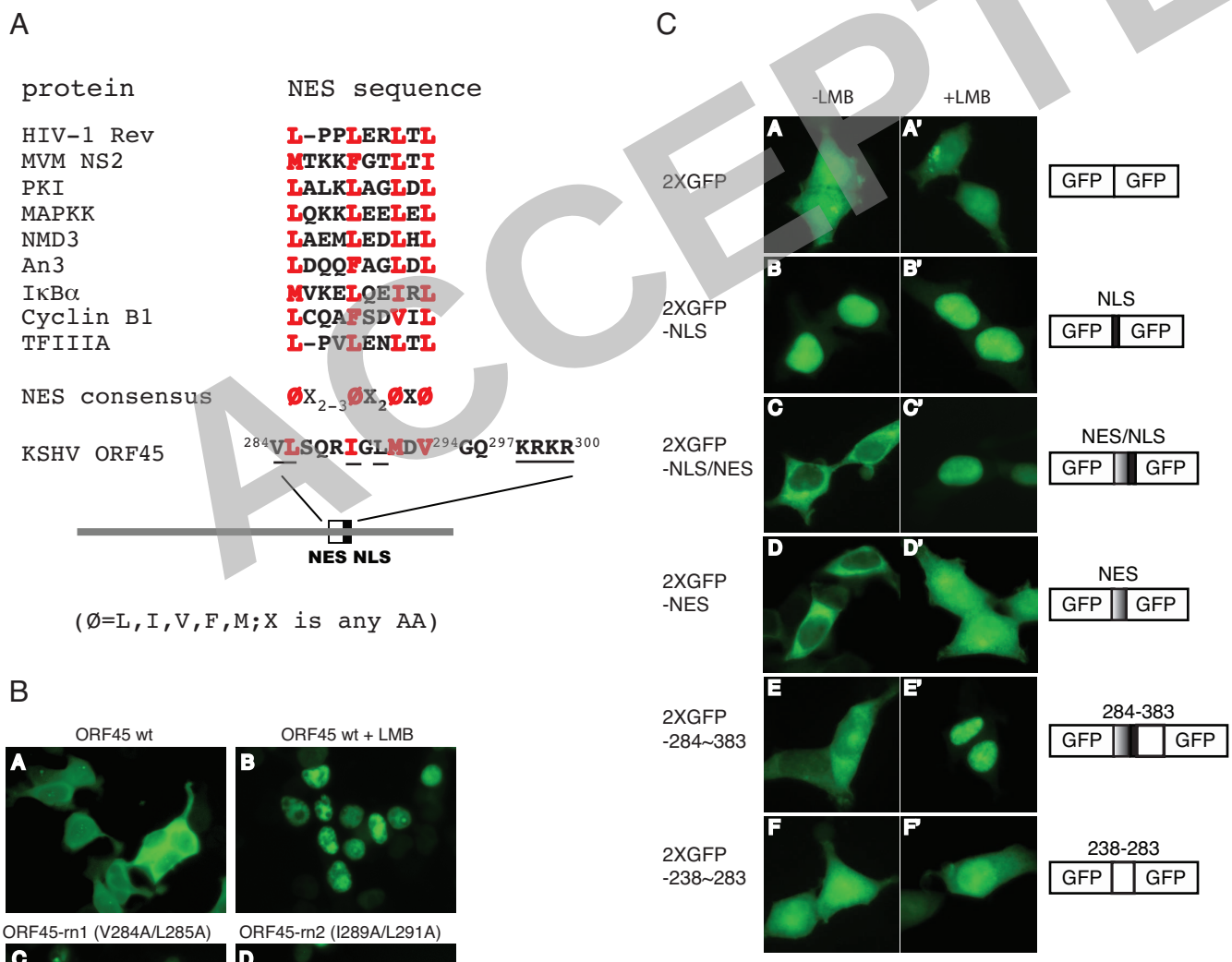


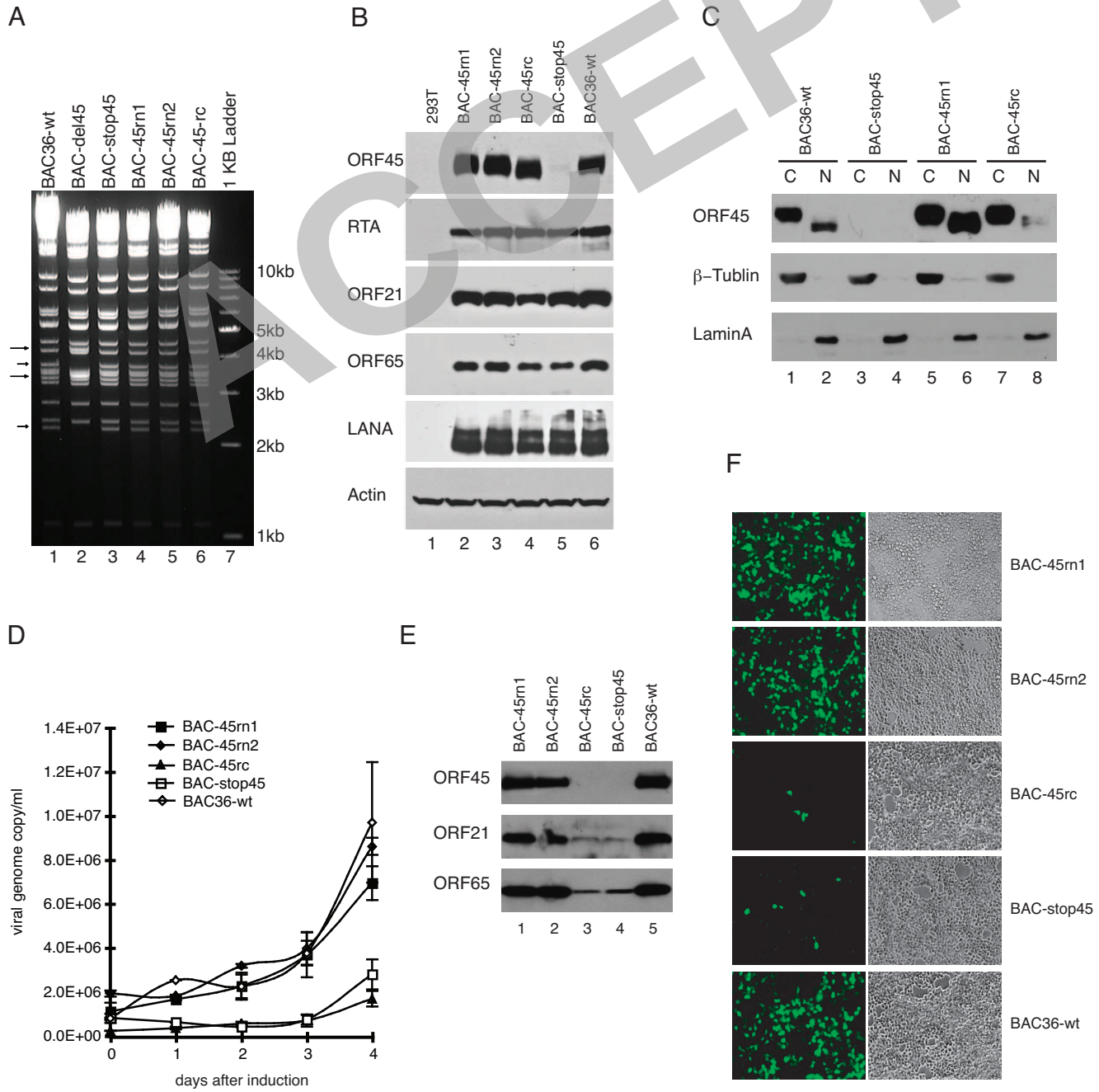
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