Kaposi sarcoma-associated herpesvirus (KSHV) ORF57 is a multifunctional, nuclear protein involved in post-transcriptional regulation of a subset of viral genes during lytic replication. Three nuclear localization signals (NLSs), NLS1 (amino acids aa 101–107), NLS2 (aa 121–130), and NLS3 (aa 143–152), were identified in the N terminus of the ORF57 protein, and each of the three represents a short stretch of basic amino acid residues. Disruption of all three NLSs prevented localization of ORF57 in the nucleus. Insertion of individual NLSs into a heterologous cytoplasmic protein converted it into a nuclear protein, confirming that each NLS functions independently and is sufficient to promote protein nuclear localization. Although it exhibits a function similar to that of Epstein-Barr virus EB2 in promoting KSHV ORF59 expression, KSHV ORF57 differs from the herpes simplex virus ICP27 protein, and its function could be disrupted by point mutations of single or two NLSs in random combination, despite the proper localization of the mutant protein in the nucleus. The dysfunctional ORF57 containing NLS mutations also had low affinity with ORF59 RNA and the RNA export factor REF. However, the REF binding of ORF57 in vivo appeared to have no effect on ORF57-mediated enhancement of ORF59 expression. Thus, the three NLSs identified in ORF57 provide at least two functions, nuclear localization of ORF57 and up-regulation of ORF59 expression.

Kaposi sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV8), is a human gammaherpesvirus that closely resembles human Epstein-Barr virus (EBV) (1). As with other gammaherpesviruses, KSHV infection is strongly associated with lymphoproliferative disorders and malignancies. It is present in all forms of Kaposi sarcoma (2, 3) and in two other diseases, body cavity-based B cell lymphoma (also known as primary effusion lymphoma) and multicentric Castleman disease (4, 5).

KSHV infection consists of two viral life cycles, as do other herpesviruses. Latent KSHV infection in Kaposi sarcoma tissues and B cell lines features the highly restricted expression of only five viral genes (6, 7). The lytic KSHV life cycle is characterized by production of progeny virus from infected cells and can be induced by chemicals such as tetradeacnol phorbol acetate and n-butylrate in primary effusion lymphoma-derived B cells with latent KSHV infection (8–10). Chemical induction in latently infected cells initiates the expression of a viral transactivator, ORF50 (RTA), which is essential for the switch from KSHV latency to the lytic phase (11, 12).

KSHV ORF57 (MTA (mRNA transcript accumulation)) is a viral early gene that encodes a nuclear protein of 455 amino acid (aa) residues (13, 14). KSHV ORF57 is a homolog of herpes simplex virus (HSV) ICP27 (15, 16), herpesvirus Saimiri (HVS) ORF57 (17–19), EBV SM or EB2 protein (20, 21), human cytomegalovirus (CMV) UL69 (22), and varicella-zoster virus ORF4 (23). Among these, ICP27 is a prototypical protein that interacts with RNA, inhibits host cell RNA splicing, and mediates nuclear export of intronless viral RNA (24–27). Although the function of KSHV ORF57 is poorly understood, several lines of evidence suggest that it is involved in the regulation of KSHV gene expression. Similar to its homologs, KSHV ORF57 increases the accumulation of mRNA targets, particularly in the cytoplasm but has no global inhibitory effects on the expression of intron-containing viral genes (13, 14). Through interactions with the RNA export factor Aly/REF (REF in this context), KSHV ORF57 has been thought to mediate nuclear export of mRNA targets in a CRM1-independent manner (28). Consistent with this hypothesis, HSV ICP27, EBV EB2, and HVS ORF57 all bind viral RNAs and mediate viral RNA export (18, 27, 29–31), presumably also through interactions with REF (31–33). In contrast, a recent study in Caenorhabditis elegans demonstrated that animals with simultaneous deletion of all three REF proteins had no defects in mRNA export, suggesting that REF proteins are not necessary for mRNA export (34). The mechanism by which KSHV ORF57 is translocated from the...
Characterization of KSHV ORF57 NLS Motifs

cytoplasm into the nucleus to perform its nuclear roles has never been explored carefully. To provide further insight into KSHV ORF57 function, we identified three nuclear localization signal (NLS) motifs in the N-terminal half of the protein that are necessary for its nuclear import. More importantly, these three NLS motifs were found to play additional important roles in ORF57-mediated enhancement of KSHV ORF59 expression.

MATERIALS AND METHODS

Plasmid Construction—To express the KSHV ORF57-GFP fusion protein in mammalian cells, a KSHV ORF57 cDNA from nt 82069 to 83541 in the KSHV genome was amplified by RT-PCR from JSC-1 cells, a KSHV+ and EBV+ B cell line (35), using a 5' primer (oVM68, 5'-TACCTGAGATTCACC/ATGTT-GACGACCGCATCCATGG-3') in combination with a 3' primer (oVM70, 5'-ATCGTGGATCC/GAAAGTGGATAAAGAATAAACCCTTG-3'). The cDNA without an intron was cloned in-frame at the GFP N terminus in the mammalian expression vector pEGFP-N1 (Clontech) via its EcoRI and BamHI sites. The resulting plasmid was named pVM8.

Various ORF57 mutants containing N-terminal truncations were created from plasmid pVM8 by PCR amplification using different 5' primers positioned at various locations in combination with a common 3' primer, oVM70. To optimize ORF57 expression in mammalian cells, all 5' primers used for N-terminal truncation of ORF57 had an introduced EcoRI site, a Kozak sequence, and an ATG start codon. The following plasmids were constructed to encode truncated ORF57s: plasmid pVM21 for aa 31–455, plasmid pVM37 for aa 112–455, plasmid pVM31 for aa 137–455, and plasmid pVM20 for aa 167–455.

ORF57 mutants containing C-terminal truncations were constructed with a similar strategy, but using a common 5' primer, oVM68, in combination with different 3' primers. The following plasmids were constructed to encode truncated ORF57s: plasmid pVM29 for aa 1–251, plasmid pVM30 for aa 1–173, plasmid pVM39 for aa 1–137, and plasmid pVM38 for aa 1–118. To create point mutations in the potential ORF57 NLS motifs, a set of two primers with a mutated target sequence was designed and used in combination with the 5' primer oVM68 or the 3' primer oVM70 for individual PCRs. The combined PCR products were further amplified by an overlapping PCR (36). Amplified products of the expected size were then cloned into the pEGFP-N1 vector as described above. The following primers were used to introduce point mutations in the ORF57 NLS motifs: oVM76 (5'-AGC-TACGGTAAATAACATCGGTGAGTTGGACAAGGCCGGGT-3') and oVM77 (5'-AAACCTCGATTTATACCAGTCGTGTTACTGTGAG-3') for the NLS1 point mutation in plasmid pVM10; oVM74 (5'-ATGACTTCTCAGGGCGAACCGGCGCTCAAATACG-3') and oVM73 (5'-GGCGGACTGATTGCCTGGACTGTAATGGGAGGA3') for the NLS2 point mutation in plasmid pVM11; and oVM102 (5'-GACGGTCGACCCCGACAGAAGGCAGCGACCGTCCGAGTTCAATTCG-3') and oVM103 (5'-GTGTCGACCTGTTCGTCCCGTACCGGCAGCTCACAGTTGGAGACCCTTTAGTG-3') for the NLS3 point mutation in plasmid pVM33. To generate a construct with point mutations in two of the three or in all three NLS motifs, the same approach was applied to generate the following constructs: plasmid pVM12 contains point mutations in both NLS1 and NLS2 and was derived from plasmid pVM10, plasmid pVM34 contains point mutations in both NLS1 and NLS3 and was also derived from plasmid pVM10, plasmid pVM36 has point mutations in both NLS2 and NLS3 and was derived from plasmid pVM11, and plasmid pVM35 has point mutations in all three NLS motifs and was derived from pVM12.

To construct a truncated ORF57 (aa 1–251) with a FLAG tag on its C terminus with or without point mutations in the NLS motifs, pVM24 (wild type) and its mutant derivatives, pVM45 (mtNLS1), pVM46 (mtNLS2), pVM47 (mtNLS3), pVM48 (mtNLS1+2), pVM49 (mtNLS1+3), pVM50 (mtNLS2+3), and pVM51 (mtNLS1+2+3) were constructed by PCR from full-length ORF57-GFP vectors with corresponding NLS mutations. The resulting PCR products were cloned into a pFLAG-CMV-5.1 vector (Sigma) via the EcoRI and BamHI sites. Plasmid pVM68, expressing full-length ORF57 protein with 3xFLAG tags on the C terminus, was constructed by PCR from plasmid pVM8. The resulting PCR products were cloned into its EcoRI and BamHI sites into a p3xFLAG-CMV-14 vector (Sigma).

Plasmid pKY15 was derived from plasmid pCMV-ICP27 (p320 from Dr. Sandri-Goldin) by inserting the HSV-1 ICP27 coding region into the EcoRI and BamHI sites of the pFLAG-CMV-5.1 vector. This plasmid has a Kozak element immediately upstream of the ICP27 ATG and expresses ICP27 as a fusion protein with a FLAG tag on its C terminus.

To examine the capability of each ORF57 NLS to convert a cytoplasmic protein into a nuclear protein, an HPV6b E6 (6E6) protein was used. The 6E6 protein lacks a notable NLS and, when expressed as a GFP fusion in the expression vector pZM266 (37), is located mainly in the cytoplasm. To introduce individual ORF57 NLSs into 6E6, several sets of 6E6-ORF57 chimeric primers containing individual NLS sequences were used for various overlapping PCRs (36), and the resulting insertions were confirmed by sequencing. Several plasmids were created with this approach as follows: plasmid pVM40 with an insertion of ORF57 NLS1, plasmid pVM41 with an insertion of ORF57 NLS2, and plasmids pVM43 and pVM44 with an insertion of ORF57 NLS3 with (pVM43) or without (pVM44) a proline residue immediately downstream.

To express ORF59 in mammalian cells, the corresponding region (nt 95552–96739) was amplified from the KSHV genome on BAC36 DNA (38) by PCR. The PCR product was then cloned into the EcoRI and BamHI sites of the pFLAG-CMV-5.1, and the resulting plasmid pVM18 expresses ORF59 as an ORF59-FLAG fusion protein.

All PCR amplifications were carried out using proofreading Tgo DNA polymerase (Roche Applied Science), and all constructs generated by PCR were verified by sequencing.

Cotransfection and Western Blot—293 cells at 2 × 105/well were transfected with 200 ng of various testing plasmids together with 300 ng of pVM18 (ORF59-FLAG) using Lipofectamine 2000. Protein samples were collected 24 h after
cotransfection by direct lysis of the cells in 2× SDS protein-sample buffer with 5% 2-mercaptoethanol. The samples were sonicated for 1 min to shear genomic DNA, boiled for 5 min, resolved on a 4–12% BisTris-buffered NuPAGE gel (Invitrogen) in 1× MES running buffer (Invitrogen), and blotted with the following antibodies: monoclonal anti-FLAG M2 (Sigma), monoclonal anti-human β-tubulin (IgM type) (Pharmingen), monoclonal anti-GFP (Clontech), and monoclonal anti-c-Myc (Sigma).

**Transfection and Imaging**—To study the subcellular localization of ORF57-GFP fusion proteins, COS-1 cells in glass tissue culture chamber slides (BD Biosciences) were transfected with 300 ng of plasmid DNA, and 24 h after transfection were fixed with cold (−20 °C) 70% ethanol for 5 min, washed once with PBS, and mounted in Vectashield mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) for DNA staining. Cell images were collected and processed into composite figures with Adobe Photoshop version 6.0 software.

**Extraction of Cytoplasmic and Nuclear Total RNA**—293 cells (5 × 10^5)/well in a 6-well plate were transfected with 600 ng of pVM18 and 400 ng of ORF57-GFP construct or pEGFP-N1 as a control. The cytoplasmic and nuclear fractions of total RNA were prepared 24 h after transfection. Briefly, after trypsinization, cells were washed with cold PBS, transferred into a 1.5-ml tube, and centrifuged at low speed. The cell pellet was resuspended in 200 μl of buffer A (50 mM Tris, pH 8.0, 140 mM NaCl, 1.5 mM MgCl₂, 0.2% Nonidet P-40, 1 mM dithiothreitol, and 200 units of RNasin), incubated 5 min on ice, and spun 2 min at 3000 × g. The supernatant (cytoplasm) was transferred into a pre-chilled tube and used to extract cytoplasmic RNA, and the pellet was saved for the extraction of nuclear RNA by the addition of 1 ml of TRIzol Reagent (Invitrogen) for total RNA extraction.

**Northern Blot Analysis**—Cytoplasmic or nuclear total RNA (5 μg) was mixed with NorthernMax formaldehyde load dye (Ambion, Austin, TX) and denatured at 75 °C for 15 min. One microliter of ethidium bromide (1 mg/ml) was added before loading. Samples were separated in a 1% agarose-formaldehyde gel in 1× MOPS buffer, transferred by capillary action onto a nylon membrane, and immobilized by UV light. Following 1 h of prehybridization, hybridization was carried out in PerfectHyb Plus hybridization buffer (Sigma) with the addition of sheared salmon sperm DNA (0.1 mg/ml). The ORF59-FLAG mRNA was detected with a γ-32P-labeled antisense probe (2 × 10⁶ cpm; nt 95870–96171). GAPDH mRNA and U6 small nuclear RNA were separately probed with individual 32P-labeled oligonucleotide probes (γZMZ270, 5′-TGAGTCCTTCACGATACCAAA-3′, for GAPDH and αST197, 5′-AAAATGGAACGCTTACCA-3′, for U6 small nuclear RNA) as indicators of fractionation efficiency. The prehybridization and hybridizations were performed at 65 °C for 20 h, followed by one wash with 2× SSPE, 0.1% SDS solution for 5 min at room temperature, and two washes with 0.2× SSPE, 0.1% SDS for 15 min at 65 °C. Membranes were exposed to a phosphorimaging screen, and the hybridization signals were captured using a PhosphorImager Storm 860 (Amer sham Biosciences) and analyzed with ImageQuant software.

**Immunoprecipitation (IP) of ORF57-associated Proteins and RNAs**—293 cells (5 × 10⁵) transfected with 500 ng of ORF57-GFP fusion plasmid DNA were harvested 30 h after transfection into 1 ml of cold IP buffer (PBS, 0.5% Triton X-100) with the addition of Complete Mini EDTA-free Protease Inhibitor Mixture (1×, catalog number 1836170; Roche Applied Science) and phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM β-glycerophosphate). The samples were incubated 5 min on ice, briefly sonicated, and centrifuged for 10 min at 10,000 × g at 4 °C. An equal amount (∼800 μg) of each extract was pre-cleared with 100 μl of pre-washed protein A/G-agarose beads (Upstate Biotechnology, Inc., Waltham, MA) for 1 h at 4 °C. At the same time, 2.5 μl of polyclonal anti-GFP antibody (Clontech) in 300 μl of IP buffer was added to 100 μl of pre-washed protein A/G-agarose beads and incubated for 1 h at 4 °C. The pre-cleaned cell extract was added to the anti-GFP antibody-coated beads and incubated for 4 h at 4 °C. The beads were then washed five times at 4 °C with 1 ml of IP buffer, and the proteins on the beads were eluted into 70 μl of 2× SDS protein sample buffer with 5% 2-mercaptoethanol. Twenty microliters of each sample was blotted first with a monoclonal anti-REF antibody (11G5, Novus Biologicals, Littleton, CO) and then reprobed with a monoclonal anti-GFP antibody (JL-8, Clontech).

IP of ORF57-associated ORF59 RNA from butyrate-activated JSC-1 cells was conducted using a polyclonal rabbit anti-ORF57 antibody. Briefly, JSC-1 cells (1 × 10⁷) induced by butyrate (3 mM) were irradiated in a 10-cm Petri dish under UV light with a dose of 480,000 μJ/cm². After removing the residual PBS from the cell pellet, the irradiated cells were then directly lysed into 400 μl of RIPA buffer and sonicated briefly. The cell lysates containing protein-RNA complexes were pre-cleaved with preimmune rabbit serum-coated beads before being used for IP with anti-ORF57 antibody-coated beads. After extensive washes, the protein-RNA complexes in the immunoprecipitates were extracted with phenol/chloroform/isoamyl alcohol and the purified RNAs were analyzed by RT–PCR for ORF59 RNA using the primer pair oST179 (5′-TAATACGACTCACTATAGG/G-TCACCTGATTGCGACGC-3′) and oST180 (5′-AACGCATCGAG/CGGTGACTTGTCGTCGACGC-3′).

To examine the ORF57-ORF59 RNA interaction in vitro, 200 μg of total cell protein containing FLAG-tagged ORF57 with or without point mutations in its NLS motifs expressed from 293 cells or 500 ng of baculovirus-expressed, FLAG-tagged ORF57 were immobilized onto anti-FLAG antibody-coated beads (EZview Red Anti-FLAG M2 Affinity Gel, Sigma) and then incubated with a pre-mixture of 4 ng of 32P-labeled ORF59 (∼1.1 kb), PAN (∼1 kb), K5 (∼0.8 kb), or luciferase RNA (∼1.1 kb) synthesized by in vitro transcription in the presence or absence of 35 μg of 293 cell nuclear extracts (NE) in 1× splicing buffer that had been mixed for 30 min on ice. Five hundred microliters of IP buffer were added, followed by 1 h of incubation at room temperature.

3 V. Majerciak and Z. M. Zheng, unpublished data.
characterization of kshv orf57 nls motifs

putative motifs in kshv orf57. p, tandem ck2 phosphorylation sites; rs, arginine-serine or serine-arginine dipeptides; arm, arginine-rich motif; rr, arginine-glycine box.

with gentle shaking. after extensive washes, protein–rna complexes remaining on the beads were resuspended in scintillation solution (cyto-scint, mp, irvine, ca), and the radioactivity from the complexes was counted in a β-counter. three independent readings were determined for each sample, averaged, and adjusted by the amount of orf57-flag fusion immobilized on the anti-flag beads as quantified by western blot with 10 μl of the beads using an anti-flag m2 antibody.

depletion of ref from 293 ne by ip and knockdown of ref expression from he la cells by sirnas—ref or hn rnnp k proteins were depleted from 293 ne by ip using anti-hrn np k (d-6, santa cruz biotechnology, santa cruz, ca) or anti-ref antibodies. the same amount of nonrelated anti-HPV16E7 antibody (santa cruz biotechnology) was used as a mouse IgG control for depletion. the ne after depletion was then tested in an orf57 RNA IP study using beads coated with a truncated form of orf57-flag fusion as described above.

to knock down ref expression from he la cells or 293 cells with a siRNA approach, 293 cells at 5 × 10⁵ per well or he la cells at 3 × 10⁵ per well were plated in a 6-well plate 1 day before transfection with 20 nM siRNA. the ref-specific siRNA duplex was purchased from Dharmaco as a SMARTpool against the THOC4 transcript (gene id 10189). the siRNA duplex 198 complementary to HPV16E7 (39), also from Dharmaco, was used as a nonspecific siRNA control. twenty-four hours after transfection, the cells in each well were split equally into two wells and cotransfected after attachment one more time with each siRNA (20 μM) into a mixture of orf59 (pVM18) (3 μg/well) and GFP (pEGFP-N1 or pVM8, an ORF57-GFP expression vector) expression vectors (2 μg/well). a reduced amount of each plasmid with the same ratio was used in 293 cell cotransfections. protein samples were collected 48 h after transfection by direct lysis of cells in 2× SDS protein sample buffer (500 μl for 293, 300 μl for HeLa) with 5% 2-mercaptoethanol and blotted using anti-FLAG M2 for ORF59, anti-GFP (clontech) for GFP and ORF57-GFP fusions, and anti-β-tubulin and anti-hnRNP K as loading controls.

results

putative motifs in the KSHV ORF57 protein—A protein sequence analysis showed that the 455-aa protein encoded by ORF57 contains multiple putative motifs (Fig. 1) and consists of over 30% basic/nucleophilic residues, most of which are clustered at the N terminus. the N-terminal half of ORF57 features a tandem array of four putative casein kinase II (CK2) phosphorylation sites (aa 20–39), four RS or SR dipeptides (aa 77–95), and a run of arginine residues (PPEKPRRRPRDRLQYG; aa 122–130). protein regions enriched in RS dipeptides and/or in arginine residues frequently harbor nuclear localization signals (37, 40, 41). in addition, the ORF57 protein contains two RGG boxes (aa 138–140 and aa 372–374) that might be involved in the RNA binding of the protein, as has been shown for other RNA-binding proteins (27, 29, 42).

the N-terminal Half of KSHV ORF57 Contains Nuclear Localization Signals—Because KSHV ORF57 has been shown by other laboratories to be a nuclear protein, we decided to determine the intrinsic properties of ORF57 that dictate its nuclear localization. a full-length cDNA of KSHV ORF57 obtained from butyrate-induced JSC-1 cells was cloned in-frame in the N terminus of GFP in a pEGFP-N1 vector. the resulting plasmid, pVM8, expresses a 455-aa ORF57-GFP fusion protein (Fig. 2, A and B). although the ORF57 cDNA construct from JSC-1 cells has one nucleotide change (T to C) at nt 82959 in the virus genome as that noticed from BCBL-1 cells (14), the single-base mutation is silent and does not result in the translation of a different amino acid. when expressed in 293 cells or COS-1 cells, the full-length, wild type (wt) ORF57 fusion protein showed exclusive nuclear localization with typical speckle-like distribution in the nucleus (Fig. 2C, wt), whereas GFP alone displayed a diffuse signal in both the nucleus and the cytoplasm (Fig. 2C, GFP). we then used the plasmid pVM8 as a template for further mutational analysis of ORF57. to map the part of ORF57 that is responsible for the nuclear localization, we constructed a series of ORF57 deletion mutants that were linked in-frame to the N terminus of GFP. we made successive deletions in the 455-aa ORF57, beginning at the N terminus and progressing to the C terminus or vice versa (Fig. 2A). all mutants (mt) with deletions were examined in COS-1 and 293 cells. the expression of the mt proteins was examined by immunoblotting, and their intracellular distributions were documented by fluorescent microscopy. as shown in Fig. 2, all constructs expressed the fusion proteins (Fig. 2B) and were present in the nucleus (Fig. 2C), except for the mt ORF57-(167–455), which localized predominantly to the cytoplasm. the mt ORF57-(137–455) differs from mt ORF57-(137–455) by only 30 residues, and the latter is localized mainly to the nucleus, suggesting that the putative NLS resides in the region of aa 137–166. in addition, mt ORF57-(1–118) differs from mt ORF57-(1–137) by 19 residues, including an arginine-rich motif that was predicted to be a potential NLS (Fig. 1, ARM), yet these two proteins displayed a similar nuclear distribution pattern, suggesting that a region upstream of the arginine-rich motif also functions as an NLS. together, data from this study indicate that the N-terminal half of the ORF57 (aa 1–167) contains multiple regions important for nuclear localization. the N-terminal half of ORF57 may also harbor a determinant for nuclear speckle-like distribution. notably, several of the mt proteins, including mt ORF57-(31–455), ORF57-(112–455), ORF57-(1–251), and ORF57-(1–173), had a speckle-like distribution in the nucleus, like the WT, but others did not (Fig. 2C), suggesting that the region from aa residue 112 to 173 in ORF57 is involved.

identification of three NLS Motifs in the N-terminal Half of KSHV ORF57 by Point Mutation Analysis—To confirm whether the N-terminal half of ORF57 contains active NLS sequences and to better define the residues responsible for nuclear localization, we analyzed the amino acid composition
within the N-terminal half of ORF57. Several regions within aa 31–167 were revealed as potentially harboring NLS-like motifs. Motif 1 runs from aa 101 to 107 and consists of 7 residues, RYGKKIK, with three lysines and one arginine; motif 2 runs from aa 121 to 130 and consists of 10 residues, KRPRRPRDR, with six arginines and one lysine; and motif 3 runs from aa 143 to 152 and is composed of 10 residues, RAAPKRATRR, with four arginines and one lysine (Fig. 3A). Collectively, all of these potential NLS-like motifs are enriched with positively charged basic residues similar to the previously reported NLS sequences in other viral and nonviral proteins (37). We thus designated these motifs NLS1 (aa 101–107), NLS2 (aa 121–130), and NLS3 (aa 143–152).

The functional characterization of the three putative NLS motifs was approached by introducing point mutations that converted basic aa residues to acidic, neutral, or small amino acid residues (Fig. 3A). As shown in Fig. 3B, the introduction of point mutations into either one or all three potential NLS
Characterization of KSHV ORF57 NLS Motifs
motifs did not change the stability or solubility of ORF57, as all ORF57 mutants were expressed well at similar levels. Most unexpectedly, mutation of a single NLS motif or in two NLSs by random combination failed to effectively prevent the nuclear import of the ORF57-GFP fusion. Although a fusion protein containing point mutations in both NLS1 and NLS2 was distributed to some extent in the cytoplasm, the majority of the protein was nuclear (Fig. 3, C and D, mtNLS1+2). Only the protein with point mutations in all three NLS motifs localized exclusively to the cytoplasm (Fig. 3, C and D, mtNLS1+2+3). Together, these data suggest that a single NLS motif is sufficient for nuclear import of the fusion protein.

Individual KSHV ORF57 NLS Sequence Motifs Promote the Nuclear Localization of the Normally Cytoplasmic HPV6 E6 (6E6) Protein—To further verify that each of these three NLS motifs is sufficient for nuclear localization of the chimeric 6E6 showed the same distribution pattern as seen in COS-1 cells (data not shown).

The NLS Motifs in KSHV ORF57 Are Essential for the Promotion of KSHV ORF59 Expression—Having identified the three NLS motifs in the N-terminal half of ORF57, we attempted to correlate the NLS-mediated nuclear localization with other functions of the ORF57 protein. A recent study reported that KSHV ORF57 is a post-transcriptional regulator that promotes the expression of KSHV ORF59 and nut-1 in transient transfection assays (14). Accordingly, the ORF59 coding region from the KSHV genome on BAC36 DNA (38) was placed under control of the CMV IE promoter as a FLAG-tagged fusion protein with the FLAG tag at the C terminus. Because KSHV ORF57 does not regulate the CMV promoter (14), this allowed us to examine the effects of ORF57 on ORF59 expression independently of any possible effects on the ORF59 promoter. The resulting vector, pVM18,
KSHV ORF57 homologs in other herpesviruses have been characterized as multifunctional proteins, and multiple regions of each protein are involved in post-transcriptional regulation (28, 43, 44). To identify sequences in ORF57 that play a role in enhancing expression of ORF59, we cotransfected each expression vector encoding the series of truncated ORF57 proteins along with the ORF59 expression vector pVM18. As shown in Fig. 6B, deletion of either the first 30 aa residues from the N terminus or 204 (aa 252–455) or 282 (aa 174–455) aa residues from the C terminus of ORF57 also affected the enhancement of ORF59 expression (compare lane 1 with lanes 2, 6, and 7), suggesting that additional elements in both N and C terminus of ORF57 are also involved in promoting ORF59 expression. Further extension of the deletion to disrupt NLS motifs (Fig. 6B, lanes 3–5, 8, and 9) in ORF57, as expected, abolished the ability of ORF57 to promote ORF59 expression.

The NLS Motifs of ORF57 Are Important for the ORF57-Mediated Accumulation of Cytoplasmic and Nuclear ORF59 RNA—After demonstrating the involvement of the ORF57 NLS motifs in up-regulation of ORF59 expression at the protein level, we attempted to determine whether the up-regulation of ORF59 expression by ORF57 is at the RNA level. A recent report suggested that ORF57 stimulates gene expression by promoting the nuclear export of a reporter RNA via interaction with REF proteins (28). The homologs of KSHV ORF57 in other herpesviruses mediate the cytoplasmic accumulation of a subset of intronless viral mRNAs by facilitating export from the nucleus to the cytoplasm (18, 27, 30–32). Our initial results from a Northern blot of total RNA showed that KSHV ORF57 enhanced ORF59 RNA expression up to about 12-fold when transiently cotransfected (data not shown). To correlate the three NLS motifs in ORF57 with a possible post-transcriptional effect of ORF57 on ORF59 expression, we compared the accumulation of ORF59 transcripts in nuclear and cytoplasmic total RNA fractions from 293 cells cotransfected with ORF57 and ORF59. Cytoplasmic and nuclear RNAs were effectively separated during cell fractionation and RNA isolation, as indicated by the presence of precursor rRNAs (45 S), immature rRNAs (32 S), and U6 only in the nuclear total RNA (Fig. 6C). When probed with an ORF59-specific probe, the amount of ORF59 RNA in the cytoplasmic and nuclear total RNAs in the presence of WT ORF57 was found, respectively, to be roughly 10- and 14-fold higher than the corresponding levels seen in its absence (GFP-only control, compare lane 1 with lane 9 in Fig. 6C), after normalization of the signal to GAPDH for cytoplasmic and U6 for nuclear total RNA. As shown in Fig. 6C, however, the levels of both the cytoplasmic and nuclear ORF59 RNAs were proportionally reduced with the introduction of point mutations into a single NLS motif of ORF57 (Fig. 6C, compare lanes 2–4 with lane 1) and were completely reduced to the basal level when two or all three of the NLSs in ORF57 were mutated (compare lanes 5–8 with lane 1). Together, these results demonstrate that the ORF57 NLS-dependent stimulation of KSHV ORF59 protein expression is at the post-transcriptional level.

The NLS Motifs of ORF57 Are Important for the ORF57-ORF59 RNA Interaction—To further understand the mechanism of how each NLS motif in ORF57 up-regulates ORF59 RNA levels, we performed in vivo protein-RNA interaction assays by using UV cross-linking and IP of ORF57-RNA complexes with an anti-ORF57 antibody based on the assumption that ORF57 interacts with RNAs in living cells. As shown in Fig.
Characterization of KSHV ORF57 NLS Motifs

Interaction of KSHV ORF57 with REF Depends on Intact NLS Motifs, but REF Displays Little Role in Promoting Interaction of ORF57 with the ORF59 RNA or Enhancing Expression of ORF59—We next examined a whole panel of mt ORF57-GFP fusions with point mutations in individual NLS motifs for their interactions with REF protein, an RNA export factor that has been hypothesized to mediate export of spliced and unspliced mRNAs from the nucleus (45). A previous study showed that KSHV ORF57 interacts directly with REF for promoting RNA export (28), and the region of ORF57 that interacts strongly with REF was mapped to a region of ORF57, aa 1–215, that encompasses the three NLS motifs identified in this study. We hypothesized that the NLS motifs of ORF57 are not only essential for accumulation of ORF59 RNA transcripts but are also important for the ORF59 RNA-ORF57 interaction. This RNA-protein interaction was specific, because ORF57 expressed from 293 cells did not show significant binding to other RNAs, including KSHV PAN and K5 and luciferase (Fig. 7E).

Involvement of the ORF57 NLS motifs in ORF59 expression. 293 cells were cotransfected with various ORF57 constructs with or without NLS point mutations (A) or N- or C-terminal truncations (B) plus an ORF57 expression vector, pVM18. pVM18 expresses ORF57 as a FLAG-tagged protein. Individual ORF57-GFP fusion or GFP-only expression vectors in the cotransfection are shown on the top of each panel. See the corresponding plasmids in Fig. 2 and Fig. 3. The protein samples were blotted with anti-FLAG (1:2500) for ORF59 24 h after transfection and re-probed with anti-GFP for fusion expression or anti-β-tubulin (1:1000) for sample loading. *, unexpected cleavage products of the fusion protein. The relative level (%) of ORF59 expression from each cotransfection was quantified from the gel on the top after normalization to the amount of the fusion protein. Both A and B are one representative of three repeats. C, a functional role of the defined NLS motifs on ORF57-mediated accumulation of ORF59 mRNA. Fractionated cytoplasmic (C) and nuclear (N) total RNAs were isolated from 293 cells 24 h after cotransfection of pVM18 along with pEGFP-N1 or individual ORF57-GFP vectors. See each corresponding plasmid in Fig. 3C. An equal amount (5 μg) of each RNA was separated in a 1% agarose-formaldehyde gel, stained with ethidium bromide (upper panel), and analyzed by Northern blot for ORF59, GAPDH, and U6 RNA transcripts. Sample loading in each lane was normalized to GAPDH RNA for cytoplasmic or to U6 RNA for nuclear total RNA and was factored into the calculation for ORF59 induction by individual ORF57 fusions. The ORF59 RNA levels in the cytoplasmic (C-RNA) or nuclear (N-RNA) fractions were expressed in bar graphs as a relative ratio (%) to ORF57 with WT NLS motifs. The panel is one representative of two repeats.

7, A and B, we were able to detect ORF59 RNA, but not cellular IL-6 RNA (data not shown), by RT–PCR from the immunoprecipitated ORF57 protein-RNA complexes. ORF59 was detected strongly in butyrate-induced but only marginally in uninduced, UV-irradiated JSC-1 cell preparations, suggesting that ORF57 is indeed specifically associated with ORF57 transcripts during lytic KSHV infection.

To explore whether the characterized NLS motifs of ORF57 are involved in the protein-RNA interactions, an in vitro ORF57 RNA-ORF57 protein interaction assay was further assessed using a truncated ORF57 with a deletion of the C-terminal half (aa 252–455). The truncated aa 1–251 ORF57-GFP fusion contains an intact N terminus encompassing all three NLS motifs as a nuclear protein and retains ~18% of its activity in promoting ORF59 expression (Fig. 6B, lane 6). However, when fused with a FLAG tag at its C terminus to replace GFP, the truncated ORF57 retained ~90% of the activity of full-length ORF57 in promoting ORF59 expression (data not shown). We then introduced the previously described point mutations into the individual NLS motifs (Fig. 3A) and expressed the mutant proteins as FLAG-tagged fusions in 293 cells by transient transfection. The expressed fusion proteins were immobilized onto anti-FLAG antibody-coated beads and examined for their binding activity to 32P-labeled ORF59 transcripts in the presence of 293 cell NE because baculovirus-expressed ORF57 itself did not show much RNA binding activity in the absence of 293 NE (Fig. 7C, compare lane 1 and lane 2). The truncated ORF57-FLAG fusion with all three NLSs intact had slightly lower activity than full-length ORF57 in ORF59 RNA binding (data not shown) but showed ~6-fold more RNA binding activity than FLAG alone (compare lanes 1 and 2 in Fig. 7D). Introduction of point mutations into each NLS motif, particularly NLS2, reduced ORF57 RNA binding activity, and further reductions in RNA binding were seen with mutation of two or three of the NLS motifs (Fig. 7D). These data strongly suggest that the characterized NLS motifs of ORF57 are not only essential for accumulation of ORF59 RNA transcripts but are also important for the ORF59 RNA-ORF57 interaction. This RNA-protein interaction was specific, because ORF57 expressed from 293 cells did not show significant binding to other RNAs, including KSHV PAN and K5 and luciferase (Fig. 7E).
Characterization of KSHV ORF57 NLS Motifs

with another report (28). However, mt ORF57-GFP fusions that contained point mutations in one, two, or all three NLS motifs showed a significant reduction in REF binding, with mutations in NLS1 being the most prominent (16-fold less than WT ORF57, compare lane 2 with lane 1). Compared with NLS2 and NLS3, which showed, respectively, a 6- and 3-fold reduction in REF binding along with the introduction of point mutations (Fig. 8A, lanes 3 and 4), the NLS1 motif with point mutations in mt ORF57-GFP fusion had only background REF binding, comparable with that seen with GFP only (compare lane 2 with lane 9), whereas the same mutation retained a substantial ability of ORF57 to promote ORF59 expression (see lane 2 in both Fig. 6, A and C), suggesting that the interaction of ORF57 with REF does not fully account for the ability of ORF57 to function in the promotion of ORF59 expression. Furthermore, although point mutations in two or all three NLS motifs led to defects in the up-regulation of ORF59 expression by ORF57 (Fig. 6, A and C), the combinations of mutations in ORF57 did not show much additional reduction in REF binding (Fig. 8A, lanes 5–8), further supporting this assumption. In fact, all three NLS motifs in ORF57 appear to interact with REF in living cells, although with different affinity. Other region(s) of ORF57 outside of the three defined NLS motifs also appeared to weakly interact with REF (Fig. 8A, compare lane 8 with lane 9). Because the mt ORF57 with all three NLS motifs disrupted is exclusively cytoplasmic (Fig. 3, C and D, pVM35), the weak interaction between REF and the mt ORF57, if any, must be in the cytoplasm.

To further test the relationship between ORF57 function and REF binding, we tested the interaction of the FLAG-tagged N-terminal half of ORF57 with ORF59 RNA in the presence or absence of REF in 293 NE. The N-terminal half of ORF57 (aa 1–251) was chosen because it retains all NLS motifs and interacts as well with REF (data not shown) and ORF59 RNA (Fig. 7) as the full-length ORF57 in the presence of 293 NE. Human hnRNP K was used as a control in this study. As shown in Fig. 8, B and C, an efficient IP depletion of both REF and hnRNP K from 293 NE, although anti-hnRNP K appeared to partially cross-react with REF, had no significant effect on the RNA binding activity of the N-terminal half of ORF57, suggesting that REF plays little role in the ORF57-ORF59 RNA interaction.

We also knocked down REF expression using siRNA to examine whether REF plays a role in ORF57-mediated ORF59 expression in HeLa and 293 cells. HeLa cells treated with REF-specific siRNA showed up to ~40% knockdown of REF expression but had no effect on hnRNP K and β-tubulin expression or on introduced GFP expression (Fig. 8D). Notably, cotransfection of the REF siRNA-treated cells with ORF59 and ORF57 expression vectors did not provide any evidence that a reduction of REF in the cells could interfere with the ability of ORF57 to enhance ORF59 expression (Fig. 8D, compare lane 4 with lane 3). A similar result was obtained in 293 cells (Fig. 8D, compare lane 8 with lane 7). Thus, the data provide further evidence that REF is not directly involved in the ORF57-mediated enhancement of ORF59 expression.

**DISCUSSION**

In this study, we have demonstrated that the nuclear localization of ORF57 is dictated by three individual NLS motifs positioned in the N-terminal half of the protein. All of these NLS motifs are enriched in basic residues, and simultaneous deletion or point mutation of the three motifs disrupts the nuclear localization of ORF57. In addition, each of the NLS sequence motifs functions independently in converting the normally cytoplasmic protein HPV6 E6 into a nuclear protein.
Moreover, we have demonstrated that these defined NLS sequence motifs in KSHV ORF57 also participate in promoting the accumulation of KSHV ORF59 RNA both in the nucleus and in the cytoplasm, perhaps through their interaction with ORF59 RNA.

Several NLS motif sequences have been identified in ORF57 homologs of other herpesviruses, and a common feature of these NLS motifs is their characteristic position in the N-terminal half of the protein (Fig. 9A). However, their structures and amino acid sequences are rather diverse, and their nuclear localization functions overlap with other activities of the protein, as we described in this study. In HSV-1 ICP27 and EBV EB2, two NLS motifs have been characterized as a bipartite signal; each motif functions independently for nuclear localization of the protein, although two are more efficient (31, 46, 47). In HSV-1 ICP27, the nuclear localization function of the arginine-rich NLS motif overlaps with its RNA-binding function (27, 29, 42) and interacts with SRPK1 to promote relocation of SRPK1 to the nucleus (48). In the EBV EB2 protein, nuclear localization and RNA binding of the protein are conferred by two completely separate sequence motifs. A 33-amino acid sequence downstream of the NLS motifs (aa 190–223) has the structural features of an arginine-rich motif and is responsible for the binding of EB2 to RNA but bears no nucleocytoplasmic shuttling activity (31). In HVS ORF57, an arginine-rich NLS motif has been characterized as the sole motif required for nuclear localization of the protein and interacting with importin α1 and α5 (49).

KSHV ORF57 and its homologs in other herpesviruses have been characterized as post-transcriptional regulators. One of their functions during viral infection is to promote the cytoplasmic accumulation of specific viral mRNAs (14, 29, 50). KSHV ORF57 has been reported to enhance the expression of KSHV FIGURE 8. REF in association with ORF57 NLS motifs plays little role in ORF57-mediated enhancement of ORF59 expression. A, point mutations in the defined NLS sequence motifs affect the interaction of ORF57 with REF protein. Cell extracts obtained from 293 cells transfected with individual GFP/ORF57 fusion plasmids with or without point mutations in the NLS motifs of ORF57 were immunoprecipitated with a polyclonal anti-GFP antibody. See corresponding plasmids in each phase image in Fig. 3C. Plasmid pEGFP-N1 was used as a GFP control. The immunoprecipitated proteins were blotted with anti-REF (1:1000) (top panel) and reprobed with anti-GFP (lower panel) for sample loading. *, unexpected cleavage products of the fusion protein. The amount of REF coimmunoprecipitated with each ORF57 fusion was quantified from each lane based on the band intensity, normalized with the amount of each GFP fusion protein coimmunoprecipitated, and expressed as a ratio (%) to WT ORF57. The figure is one representative of two repeats. B and C, depletion of REF from 293 NE does not affect the interaction between the N-terminal half of ORF57 and ORF59 RNA. REF and hnRNP K were depleted by anti-REF and anti-hnRNP K IP from 293 NE. Anti-HPV16 E7 was used as an antibody control for IP. The depleted NE was then analyzed by Western blot for depletion efficiency (B) and utilized for RNA binding assays (C). The inset in C shows the ORF57-FLAG bound on the beads for RNA binding assays and was blotted from 10% of the total anti-FLAG-coated beads immunoprecipitated with the N-terminal half ORF57-FLAG fusion expressed from 293 cells. D, down-regulation of REF by siRNA does not affect ORF57-mediated ORF59 expression. HeLa and 293 cells pretreated once with REF-specific siRNA or nonspecific (NS) HPV16 E7 siRNA were cotransfected with a mixture of mammalian expression vectors, pVM18 (ORF59-FLAG) plus pVM8 (ORF57-GFP) or pEGFP-N1 (GFP), in the presence of a second dose of individual siRNAs. The protein samples were blotted 48 h after transfection. The remaining REF in each protein sample was normalized to the hnRNP K level and expressed as a ratio (%) to the corresponding NS siRNA control.
ORF59 at the post-transcriptional level (14). Using ORF59 as a target for ORF57, we demonstrated that both KSHV ORF57 and EBV EB2 displayed a similar function in the promotion of ORF59 expression but HSV ICP27 did not, indicating that both KSHV ORF57 and EBV EB2 are also functionally distinct from ICP27. Interestingly, the defined NLS sequence motifs in ORF57 were found to be associated with enhancement of ORF59 expression by ORF57. Disruption of any one of the three NLS motifs in ORF57 by point mutation led to a partial reduction of ORF57-mediated nuclear and cytoplasmic accumulation of ORF59 RNA and protein expression, despite not resulting in any defect of protein import. Further disruption of any two of the three NLS motifs in ORF57 by point mutation almost completely abolished the ability of ORF57 to enhance ORF59 RNA and protein expression, despite the fact that one intact NLS motif is sufficient for the nuclear import of ORF57. Because ORF57 with NLS mutations displayed a nuclear defect in addition to a reduction in cytoplasmic accumulation of ORF59 RNA, we propose that KSHV ORF57 might function at a step prior to RNA export in stimulation of ORF59 expression.

Although it remains to be understood how the NLS motifs function in promoting the accumulation of ORF59 transcripts, our study suggests that it involves an interaction with ORF59 RNA, independently from its interaction with REF, a nuclear export factor. Although the identified NLS motifs in ORF57 are important for REF binding, the ORF57-REF interaction through the NLS motifs appears to play at best a limited role in promoting ORF59 expression. Another study has demonstrated that the N-terminal half of KSHV ORF57 (aa 1–215), the region encompassing all three NLS motifs identified in this study, contains a region that interacts with REF (28). In this regard, the NLS1 motif apparently shows the greatest affinity for REF, but disruption of NLS1 in ORF57 had only a partial reduction in the ability of ORF57 to promote ORF59 expression and bind to ORF59 RNA. Moreover, our study also shows that, although both the NLS2 and NLS3 motifs are important for REF binding, disruption of any two or all three NLS motifs, which renders the ORF57 defective in promoting ORF59 expression and in ORF59 RNA binding, did not additionally reduce REF binding, suggesting that other factors besides REF might interact with the NLS motifs (Fig. 9B) and account for the ORF57-mediated enhancement of ORF59 expression. This conclusion was further supported by the observation that cells with a knockdown of REF expression had no defect in ORF57-enhanced ORF59 expression. Other studies also show no defect in RNA export in REF knock-out animals (34, 51). During the course of our manuscript preparation, Chen et al. (52) reported that HSV-1 ICP27, a homolog of KSHV ORF57, contains NLS motifs similar to those in ORF57 and interacts with REF. This study also showed that HSV-1 ICP27 is required for the nuclear accumulation of newly transcribed viral RNAs and that expression of HSV-1 ICP27 is dependent on the NLS motifs for its nuclear localization.
olog of KSHV ORF57, mediates viral RNA export through the TAP/NXF1 pathway independent of its interaction with REF.

KSHV ORF57 homologs in other herpesviruses contain other regions that interact with factors involved in RNA processing and export. In HSV-1 ICP27, which also contains two RS/SP dipeptides, a C-terminal zinc finger motif is required for the interaction of ICP27 with the N-terminal RRM of SRp20 (48). In EBV EB2, a region (aa 218–236) containing two nuclear export signal sequence motifs has been found to interact with the REF C-terminal RGG repeat region, although this region is not necessary for the nucleo-cytoplasmic shuttling of the protein (31). In HVS ORF57, several regions interact with the RNA export factor REF (ORF57 aa 8–120) or the exon junction complex components, Y14 (ORF57 aa 121–240 and aa 293–417) and Magoh (33). We have shown in this study that both the N-terminal 30 residues and the C-terminal 204 residues of KSHV ORF57, outside of the three defined NLS sequence motifs, are also partially involved in promoting ORF57 expression, because disruption of ORF57 protein integrity by deletion of these regions showed a partial effect on the function of ORF57 (Fig. 6B). Several other regions of ORF57 involved in interactions with RNA processing or translation factors have also been roughly mapped. ORF57 aa 181–215 was found to be the minimal region required for weak interaction with REF (28) and CK2β (43). The residues 17–181 and 329–387 of ORF57 are involved in hnRNP K binding, and the CK2α′ binding regions in ORF57 have been mapped to residues 387–455 (43). Moreover, an N-terminal region (residues 179–205) of KSHV ORF57 has been found to interact with PCBP1, a cellular RNA binding protein involved in internal ribosome entry site-mediated gene expression (44). It will be of great interest to know how these regions coordinate with the NLS motifs identified in this study to regulate viral RNA accumulation, processing, and export, and whether the NLS sequence motifs in KSHV ORF57 might be involved in the recruitment of protein factors that interact with other regions of ORF57.

Acknowledgments—We thank David Lukir for providing the pCMV-ICP27 expression vector (p320); and Russ Carstens for the pCMV-ICP27 expression vector (pGS113); Rozanne Sandri-Goldin for ORF57 antibody and ORF57 plasmids for our initial antibody and ORF57 (Fig. 6). Several other regions of ORF57 involved in interactions with RNA processing or translation factors have also been roughly mapped. ORF57 aa 181–215 was found to be the minimal region required for weak interaction with REF (28) and CK2β (43). The residues 17–181 and 329–387 of ORF57 are involved in hnRNP K binding, and the CK2α′ binding regions in ORF57 have been mapped to residues 387–455 (43). Moreover, an N-terminal region (residues 179–205) of KSHV ORF57 has been found to interact with PCBP1, a cellular RNA binding protein involved in internal ribosome entry site-mediated gene expression (44). It will be of great interest to know how these regions coordinate with the NLS motifs identified in this study to regulate viral RNA accumulation, processing, and export, and whether the NLS sequence motifs in KSHV ORF57 might be involved in the recruitment of protein factors that interact with other regions of ORF57.

REFERENCES

1. Chang, Y., Cesaran, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994) Science 266, 1865–1869
2. Hayward, G. S. (2003) Cancer Cell 3, 1–3
3. Viejo-Borbolla, A., Ottinger, M., and Schulz, T. F. (2003) Curr. Infect. Dis. Rep. 5, 169–175
4. Soulier, J., Groflet, L., Oksenhendler, E., Cacoub, P., Casals-Hatem, D., Babinet, P., d’Agay, M. F., Clauvel, J. P., Raphael, M., Degos, L., and Sigaux, J. A., Gao, S. J., Chang, Y., and Moore, P. (1996) Nat. Med. 2, 342–346
5. Sun, S., Lin, S. F., Gradoville, L., Yuan, Y., Zhu, F., and Miller, G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10866–10871
6. Lukir, D. M., Kirshner, J. R., and Ganem, D. (1999) J. Virol. 73, 9348–9361
7. Gupta, A. K., Ruvolo, V., Patterson, C., and Swaminathan, S. (2000) J. Virol. 74, 1038–1044
8. Kirshner, J. R., Kirshner, D. M., Chang, Y., and Ganem, D. (2000) J. Virol. 74, 3586–3597
9. Sandri-Goldin, R. M., and Hibbard, M. K. (1996) J. Virol. 70, 108–118
10. Sandri-Goldin, R. M., Hibbard, M. K., and Hardwicke, M. A. (1995) J. Virol. 69, 6063–6076
11. Whitehouse, A., Cooper, M., and Meredith, D. M. (1998) J. Virol. 72, 857–861
12. Goodwin, D. J., Hall, K. T., Stevenson, A. J., Markham, A. F., and Whitehouse, A. (1999) J. Virol. 73, 10519–10524
13. Cooper, M., Goodwin, D. J., Hall, K. T., Stevenson, A. J., Meredith, D. M., Markham, A. F., and Whitehouse, A. (1999) J. Gen. Virol. 80, 1311–1316
14. Cook, I. D., Shanahan, F., and Farrell, P. J. (1994) Virology 205, 217–227
15. Ruvolo, V., Wang, E., Boyle, S., and Swaminathan, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8825–8832
16. Sandri-Goldin, R. M. (1998) Genes Dev. 12, 868–879
17. Malik, P., Blackbourn, D. I., and Clements, J. B. (2004) J. Biol. Chem. 279, 33001–33011
18. Sokolowski, M., Scott, J. E., Heaney, R. P., Patel, A. H., and Clements, J. B. (2003) J. Biol. Chem. 278, 35340–35349
19. Ruvolo, V., Gupta, A. K., and Swaminathan, S. (2001) J. Virol. 75, 6033–6041
20. Hiriart, E., Farjob, G., Gruffat, H., Nguyen, M. V., Sergeant, A., and Manet, E., (2003) J. Biol. Chem. 278, 335–342
21. Koffa, M. D., Clements, J. B., Izaurrelde, E., Wadd, S., Wilson, S. A., Matta, I. W., and Kuersten, S. (2001) EMBO J. 20, 5769–5778
22. Williams, B. J., Bone, E. H., Goodwin, D. J., Roaden, L., Hauertbergue, G. M., Wilson, S. A., and Whitehouse, A. (2005) Biochem. J. 387, 295–308
23. Longman, D., Johnstone, I. L., and Caceres, J. F. (2003) RNA (Cold Spring Harbor) 9, 881–891
24. Cannon, J. S., Ciufo, D., Hawkins, A. L., Griffin, C. A., Borowitz, M. I., Hayward, G. S., and Ambinder, R. F. (2000) J. Virol. 74, 10187–10193
25. Zheng, Z. M., Reid, E. S., and Baker, C. C. (2000) J. Virol. 74, 10612–10622
26. Tao, M., Kruhlak, M., Xia, S., Androphy, E., and Zheng, Z. M. (2003) J. Virol. 77, 13232–13247
27. Zhou, F. C., Zhang, Y. J., Deng, J. H., Wang, X. P., Pan, H. Y., Hettler, E., and Gao, S. J. (2002) J. Virol. 76, 6185–6196
28. Tang, S., Tao, M., McCoy, J. P., Jr., and Zheng, Z. M. (2006) J. Virol. 80, 4249–4263
29. Hedley, I. M., Amrein, H., and Maniatis, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11524–11528
30. Caceres, J. F., Mistelli, T., Scroto, G. R., Spector, D. L., and Krainer, A. R. (1997) J. Cell Biol. 138, 225–238
Characterization of KSHV ORF57 NLS Motifs

42. Mears, W. E., and Rice, S. A. (1996) J. Virol. 70, 7445–7453
43. Malik, P., and Clements, J. B. (2004) Nucleic Acids Res. 32, 5553–5569
44. Nishimura, K., Ueda, K., Guwanan, E., Sakakibara, S., Do, E., Osaki, E., Yada, K., Okuno, T., and Yamanishi, K. (2004) Virology 325, 364–378
45. Rodrigues, J. P., Rode, M., Gatfield, D., Blencowe, B. J., Carmo-Fonseca, M., and Izaurralde, E. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1030–1035
46. Mears, W. E., Lam, V., and Rice, S. A. (1995) J. Virol. 69, 935–947
47. Hibbard, M. K., and Sandri-Goldin, R. M. (1995) J. Virol. 69, 4656–4667
48. Sciabica, K. S., Dai, Q. J., and Sandri-Goldin, R. M. (2003) EMBO J. 22, 1608–1619
49. Goodwin, D. J., and Whitehouse, A. (2001) J. Biol. Chem. 276, 19905–19912
50. Pearson, A., Knipe, D. M., and Coen, D. M. (2004) J. Virol. 78, 23–32
51. Gatfield, D., and Izaurralde, E. (2002) J. Cell Biol. 159, 579–588
52. Chen, I. H., Li, L., Silva, L., and Sandri-Goldin, R. M. (2005) J. Virol. 79, 3949–3961