Elimination of Mitochondrial DNA Is Not Required for Herpes Simplex Virus 1 Replication

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ABSTRACT

Infection with herpes simplex virus type 1 (HSV-1) results in the rapid elimination of mitochondrial DNA (mtDNA) from host cells. It is known that a mitochondrial isoform of the viral alkaline nuclease (UL12) called UL12.5 triggers this process. However, very little is known about the impact of mtDNA depletion on viral replication or the biology of HSV-1 infections. These questions have been difficult to address because UL12.5 and UL12 are encoded by overlapping transcripts that share the same open reading frame. As a result, mutations that alter UL12.5 also affect UL12, and UL12 null mutations severely impair viral growth by interfering with the intranuclear processing of progeny viral genomes. Therefore, to specifically assess the impact of mtDNA depletion on viral replication, it is necessary to eliminate the activity of UL12.5 while preserving the nuclear functions of UL12. Previous work has shown that the human cytomegalovirus alkaline nuclease UL98 can functionally substitute for UL12 during HSV-1 replication. We found that UL98 is unable to deplete mtDNA in transfected cells and therefore generated an HSV-1 variant in which *UL98* coding sequences replace the *UL12/UL12.5* open reading frame. The resulting virus was severely impaired in its ability to trigger mtDNA loss but reached titers comparable to those of wild-type HSV-1 in one-step and multistep growth experiments. Together, these observations demonstrate that the elimination of mtDNA is not required for HSV-1 replication in cell culture.

IMPORTANCE

Herpes simplex virus types 1 and 2 destroy the DNA of host cell mitochondria, the powerhouses of cells. Epstein-Barr virus, a distantly related herpesvirus, has a similar effect, indicating that mitochondrial DNA destruction is under positive selection and thus confers a benefit to the virus. The present work shows that mitochondrial DNA destruction is not required for efficient replication of herpes simplex virus type 1 in cultured Vero kidney epithelial cells, suggesting that this activity likely benefits the virus in other cell types or in the intact human host.

During infection of a host cell, viruses must compete effectively for limited resources while counteracting host antiviral defenses to facilitate their replication. One strategy used by human alpha- and gammaherpesviruses to facilitate such competition is to shut off the expression of host genes located in the nuclear genome. Host shutoff involves inhibition of host gene transcription (1), alterations in mRNA processing (2, 3), and the loss of cytoplasmic mRNAs (4–8). Altogether, these effects help redirect the cellular gene expression machinery toward the expression of viral genes. Interestingly, herpes simplex virus type 1 (HSV-1), HSV-2, and Epstein-Barr virus (EBV) have also evolved mechanisms to disrupt the expression of genes located in the host mitochondrial genome by degrading mitochondrial DNA (mtDNA) (9, 10).

Mitochondria are small cytoplasmic organelles that mediate diverse functions, including energy production, calcium homeostasis, antiviral signaling, intermediary metabolism, and programmed cell death (reviewed in reference 11). The majority of mitochondrial proteins which affect these functions are encoded by the nuclear genome; however, a limited subset of mitochondrial proteins is encoded by the mitochondrial genome. Residing within the mitochondrial matrix, mtDNA genomes are small (16.6-kb) double-stranded DNA (dsDNA) circles that encode 13 protein subunits of the energy-generating machinery, 22 mitochondrial tRNAs, and two mitochondrial rRNAs (12–14).

During EBV lytic infection, the Zta transactivator protein associates with the mitochondrial single-stranded DNA binding protein (mtSSB) and redirects mtSSB to the nucleus (10). As a consequence, the co-option of mtSSB is thought to facilitate EBV replication while inhibiting mtDNA replication (10). Similarly, HSV-1 and HSV-2 cause rapid and complete loss of mtDNA and mitochondrial mRNAs (mt-mRNAs) from infected cells (9, 15). In the case of HSV-1, our laboratory has demonstrated that a mitochondrially targeted viral nuclease, UL12.5, is sufficient to trigger mtDNA depletion in the absence of other HSV-1 proteins in transfected cells (9). Interestingly, our recent data indicate that the nuclease activity of UL12.5 is not strictly required for mtDNA loss and show that the host mitochondrial nucleases endonuclease G (ENDOG) and endonuclease G-like 1 (EXOG) are also involved in the depletion process (16).

UL12.5 is an amino-terminally truncated isoform of the viral alkaline nuclease (UL12) that is conserved in all herpesviruses. UL12 and UL12.5 are encoded by separate overlapping 3′ co-terminal transcripts (17, 18) that result in two proteins with similar 5′→3′ exonuclease and endonuclease activities (19–21). However, the translation of UL12.5 initiates from UL12 codon

M127, resulting in a protein that lacks the N-terminal 126 amino acid residues of UL12 (18, 22, 23). This difference in the amino acid sequences targets UL12 and UL12.5 proteins to distinct subcellular compartments and confers different functions during infection. The 126-residue UL12-specific N-terminal sequence targets UL12 to the nucleus (21, 24), where it participates in the maturation of viral DNA genomes during infection (25–27). In contrast, the N-terminal truncation of UL12.5 unmasks a mitochondrial localization signal (MLS), located near the N terminus of UL12.5, which directs UL12.5 to mitochondria, where it triggers mtDNA depletion (28).

The coding sequence for UL12.5 is completely embedded within the *UL12* gene (23); therefore, any mutation that alters the amino acid sequence of UL12.5 will also affect full-length UL12. We have previously shown that some but not all mutations that inactivate the nuclease activity of UL12.5 abolish the ability of UL12.5 to provoke mtDNA loss (16). However, because these mutations also inactivate the nuclear functions of UL12 and therefore severely impair virus replication (29), they cannot be used to cleanly assess the role of UL12.5-mediated mtDNA depletion during infection. Such an assessment would require the generation of mutations that prevent the production of UL12.5 or eliminate its ability to deplete mtDNA without affecting the function of UL12. Using an elegant approach, Martinez et al. generated a UL12.5 null mutant virus by converting the UL12.5 initiator codon at position 127 from a methionine to a phenylalanine codon (M127F) to prevent the translation of UL12.5 (22). This virus grew comparably to wild-type virus, indicating that intact UL12.5 is not required for viral replication in cell culture (22). However, we subsequently showed that the M127F mutant virus depletes mtDNA as efficiently as wild-type HSV-1 due to the expression of an mtDNA depletion-competent protein (termed UL12$_{M185}$) that initiates at the next in-frame methionine codon (M185) (28). These results therefore leave open the question of the role of mtDNA depletion in HSV-1 biology.

In this study, we demonstrate that the ability of HSV-1 UL12.5 to mediate mtDNA depletion is not conserved among UL12 orthologs of several other human herpesviruses. This observation led us to generate an HSV-1 mutant virus in which the *UL12/UL12.5* open reading frame (ORF) was functionally replaced with the alkaline nuclease-coding sequence of human cytomegalovirus (HCMV), *UL98*. This mutant virus replicated to normal titers but was severely impaired in its ability to mediate mtDNA depletion, indicating that the elimination of mtDNA is not required for HSV-1 replication in cell culture.

MATERIALS AND METHODS

Cells. Vero and HeLa cells were maintained at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 5% or 10% heat-inactivated fetal bovine serum (FBS) (Sigma), respectively. MRC-5 cells were maintained in DMEM supplemented with 5% FBS, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (Gibco). The EBV-positive cell line B95-8 was maintained in HEPES-buffered RPMI 1640 medium (Gibco) supplemented with 10% FBS and 2 mM glutamine. Cre-Vero cells, stably expressing the Cre recombinase (30), were grown similarly to Vero cells, except that 400 μg/ml hygromycin B (Invitrogen) was added at every fifth passage to maintain the transgene. The UL12-complementing cell line 6-5 (25) was maintained in complete Vero medium supplemented with 250 μg/ml G418 (Gibco). Vero cells and 6-5 cells were cultured in the presence of 100 U/ml penicillin-streptomycin (Gibco).

Primers and PCRs. The primers used are listed in Table 1 and were synthesized by Integrated DNA Technologies. All PCRs were performed using Platinum Pfx DNA polymerase (Invitrogen).

Plasmids and transfections. The pF1′-CMV-AK(M127F/M185L) expression plasmid containing a *UL12* gene with mutations in methionine codons 127 and 185 and the pUC119-AK plasmid containing a 3.2-kb SphI fragment bearing the *UL12* gene (18) were kindly provided by Sandra Weller. The plasmid pEPKan-S (31) was kindly provided by Nikolaus Osterrieder. The plasmids pMZS3F (32), encoding the sequential peptide affinity (SPA) tag, and pMZS3F UL98-SPA, encoding SPA-tagged HCMV UL98, were kindly provided by Lori Frappier. The pMZS3F UL12.5-SPA plasmid (16) and a plasmid expressing monomeric orange fluorescent protein (pcDNA-Orange) (9) have been described previously.

To generate plasmids encoding SPA-tagged UL12 orthologs, total DNA was isolated from Vero cells infected with HSV-2, MRC-5 cells infected with varicella-zoster virus (VZV), or B95-8 cells latently infected with EBV. The viral genes were amplified by PCR using the primers indicated as follows: HSV-2 *UL12* (F1 and R1), HSV-2 *UL12$_{M117}$* (F2 and R1), VZV *ORF48* (F3 and R2), and EBV *BGLF5* (F4 and R3). The plasmid pcDEF3 ORF37 (7), kindly provided by Britt Glaunsinger, was used to amplify Kaposi's sarcoma-associated herpesvirus (KSHV) *ORF37* (*SOX*) using primers F5 and R4. All *UL12* orthologs were first cloned into pcDNA3.1(-) UL12.5-SPA (16) to place the desired ORF in-frame with the SPA tag-coding sequence and then subcloned into the final expression vector, pMZS3F.

All plasmid transfections were performed in HeLa cells using Lipofectamine 2000 (Invitrogen), with the medium being changed to complete HeLa medium supplemented with 50 μg/ml uridine and 1 mM sodium pyruvate after 5 h.

Viruses. Two wild-type HSV-1 strains were used in this study: the infectious bacterial artificial chromosome (BAC)-derived strain KOS37 (30), obtained from David Lieb, and strain KOS. The HSV-2 strain HG52 was used for plasmid generation. The UL12, UL12.5, and UL12$_{M185}$ null virus AN1, which bears a deletion/insertion mutation within the *UL12* ORF, was obtained from Sandra Weller (33). VZV strain Oka-P was kindly provided by Graham Tipples.

KOS37-derived viruses KOS37 M127F/M185L (F/L), KOS37 M127F/M185L Rescue (F/L Res), and KOS37 SPA were generated in *Escherichia coli* strain SW102 using lambda Red-mediated homologous recombination (34; protocol available at http://ncifrederick.cancer.gov/research/brb/protocol/Protocol3_SW102_galK_v2.pdf). To generate the F/L BAC, a *galK* expression cassette was produced by PCR from pGalK (34) using primers F6 and R5 and inserted into the KOS37 BAC between *UL12* codons 126 and 186. Next, the expression plasmid pF1′-CMV-AK(M127F/M185L) was used to generate a PCR product using primers F7 and R6 to seamlessly replace the *galK* cassette with the desired mutated *UL12* sequence (Fig. 1). The F/L Res BAC was created from the F/L BAC using the same protocol outlined above. The *galK* expression cassette was made and inserted as indicated above and then replaced with a wild-type *UL12* PCR product amplified from pUC119-AK using primers F7 and R6 (Fig. 1). To construct the SPA BAC, the 3′ portion of *UL12* was recoded and fused to a 3′ SPA tag-coding sequence. This recoding involved placing silent mutations in 208 of 242 codons of the 3′ 726-bp portion of *UL12* (in order to avoid recombination with the wild-type *UL12* 3′ sequence; see below). The sequence of the recoded region of *UL12* is available upon request. A *galK* expression cassette amplified from pGalK using primers F8 and R7 was inserted between nucleotides 1152 and 1153 of *UL12* in the KOS37 BAC. A plasmid containing the recoded region of *UL12* fused in-frame to the SPA tag-coding sequence was synthesized by GeneArt. This synthesized *UL12* sequence was amplified using the primers F9 and R8 and used to replace the *galK* cassette, generating the SPA BAC. This manipulation placed the recoded section of *UL12* (fused to the SPA tag) upstream from the wild-type *UL12* 3′ sequence, allowing the expression of the desired SPA-tagged UL12 and UL12.5 proteins while avoiding DNA

TABLE 1 Oligonucleotides used in this study

Primer	Sequence (5′→3′)^a
F1	G**GAATTC**CGCCACCATGGCGGCCGCCGCAACACC
F2	G**GAATTC**CGCCACCATGTGGTCGGCGTCGGCGATCCCC
F3	G**GAATTC**CGCCACCATGGCACGATCGGGATTGG
F4	G**GAATTC**CGCCACCATGGCCGACGTGGATGAGCTCG
F5	G**GAATTC**CGCCACCATGGAGGCCACCCCCACACC
F6	CCCACGCCCGCGACCCGGACGCCGATCCCGACTCCCCGGACCTTGACTCTCCTGTTGACAATTAATCATCGGCA
F7	CCCACGCCCGCGACCCGG
F8	GGGCCAAGTACGCTTTCGACCCCATGGACCCCAGCGACCCCACGGCCTCCCCTGTTGACAATTAATCATCGGCA
F9	GGGCCAAGTACGCTTTCGACCCCATGGACCCCAGCGACCCCACGGCCTCCGCCTATGAAGATTTGATGGCTCATCG
F10	GCTGC**GGGCCC**ACGGTACGCCAGTAGCCGAGGACTTTATGACGCGCGTGGCCGCGTTGGCT**TAGGGATAACAGGGTAAT**CGATTT
F11	GTTCACCCGGCGGCGCGCTCAACCACCGCTCCCCCCACGTCGTCTCGGAAATGTGGGGCGTCTCGAG
F12	CCGCAGACGAAAAGCCCCGG
F13	GGGACATTCACGGCTACCTGG
F14	GCGAGTTTCTGCTTTCGCACG
R1	GG**GGTACC**GCGAGACGACCTCCCCGCCGTCG
R2	GG**GGTACC**AAGCAACGGTTTCTCCGTTGC
R3	GG**GGTACC**TGGAGTTGACTCGTCGTCGGCAAAGAG
R4	GG**GGTACC**CGGGCTGTGAGGGACGTTTGCAG
R5	GGCCCGCGGCGCGTCAGGCGCCATAGGTGCCGACCGAGTCCGCGGTCCACTCAGCACTGTCCTGCTCCTT
R6	GGCCCGCGGCGCGTCAGG
R7	AATGCCCGGAACGCCTCCGGGGACCGGTGTGCCATCAAGTCCTCGTACGCTCAGCACTGTCCTGCTCCTT
R8	AATGCCCGGAACGCCTCCGGGGACCGGTGTGCCATCAAGTCCTCGTACGCCTACTTGTCATCGTCATCCTTGTAGTC
R9	ACCGT**GGGCCC**GCCAGTGTTACAACCAATTAACC
R10	AATGCCCGGAACGCCTCCGGGGACCGGTGTGCCATCAAGTCCTCGTACGCCTACTTGTCATCGTCATCCTTGTAGTCG
R11	CCTCGTAAAACCCCATGAGGGGCC
R12	CTTGCGTGACGAGAGCCTCC
COII	GGAGTCGAAGGTCTCCTGGGTTTAAGAATAATGGGGG

^{a} HSV homology regions are underlined and restriction sites are in boldface.

sequence duplication or disruption of the expression of the overlapping UL11 gene (Fig. 1).

A UL98-expressing KOS37 BAC (KOS37 UL98) was created by using *en passant* mutagenesis in the *E. coli* strain GS1783 (35). The kanamycin resistance gene, *aphAI*, and an I-SceI restriction endonuclease site were amplified from the vector pEPKan-S using the primers F10 and R9 and cloned into the pMZS3F UL98-SPA vector using the ApaI site. The resulting *UL98* sequence, containing *aphAI*, an I-SceI site, and a sequence du-

plication, was amplified by PCR using primers F11 and R10 and targeted into the KOS37 BAC. This recombination event deleted nucleotides 1 to 1152 of *UL12*, preventing UL12, UL12.5, and UL12$_{M185}$ expression, while leaving the *UL11* gene intact. A subsequent recombination event removed the *UL98* sequence duplication, *aphAI* gene, and I-SceI site, resulting in the UL98 BAC.

All mutant BACs were screened by PCR using the primers F12 and R11 (F/L and F/L Res), F13 and R12 (KOS37 SPA), or F14 and R12 (KOS37
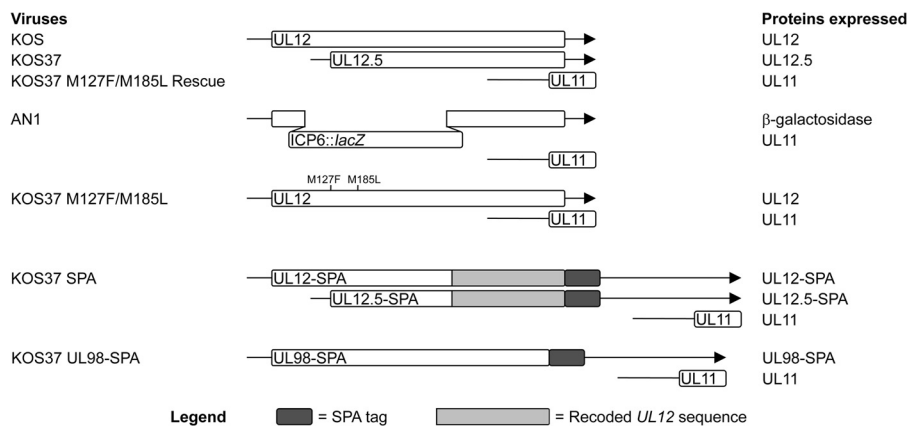

FIG 1 Viruses used to examine the effect of mtDNA depletion on HSV-1 replication. Schematics of the *UL12*, *UL12.5*, and *UL11* loci in HSV-1 mutant viruses used in this study are shown. KOS, KOS37, and KOS37 M127F/M185L Rescue (top) are wild-type viruses. AN1 possesses a partial deletion and insertion within *UL12* which eliminates UL12 and UL12.5 expression (33). KOS37 M127F/M185L contains two substitution mutations within the second and third in-frame methionine codons of *UL12* which prevent translation of UL12.5 and UL12$_{M185}$. KOS37 SPA contains silent mutations in the 3′ end of the UL12 gene which facilitate the expression of C-terminally SPA-tagged UL12 and UL12.5 proteins without affecting UL11 expression. KOS37 UL98-SPA is a UL12/UL12.5 null virus which expresses a C-terminally SPA-tagged version of the UL12 ortholog HCMV UL98 in a manner that does not affect UL11 expression. The mRNA transcripts are represented by black arrows, and the open reading frames are represented by boxes. All features are drawn to scale.

UL98). Wild-type and mutated BACs were propagated in *E. coli*, isolated using a large-construct kit (Qiagen), and transfected into Cre-Vero cells to generate infectious viral particles. Passaging through Cre-Vero cells was performed to facilitate the removal of the BAC sequence from the viral DNA.

DNA isolation and Southern blotting to detect mtDNA. Cells for DNA isolation were lysed in urea-SDS lysis buffer (7 M urea, 350 mM NaCl, 10 mM Tris [pH 7.8], 10 mM EDTA, 1% SDS). The isolated DNA was sheared using QIAshredder columns (Qiagen), phenol-chloroform extracted, and precipitated with ethanol. The precipitated DNA was resuspended in 0.3 M sodium acetate and treated with 10 mg/ml RNase A for 45 min at 37°C, followed by ethanol precipitation and resuspension in distilled H_2O.

Equivalent amounts of DNA were treated with HpaI and subjected to agarose gel electrophoresis, and then the total DNA signal was quantitated using a FLA-5100 imaging system (Fujifilm) following SYBR Gold (Invitrogen) staining. The gel was washed sequentially with 0.25 M HCl, 0.5 M NaOH, 1 M Tris (pH 7.4)–1.5 M NaCl, and 150 mM sodium citrate (pH 7.0)–1.5 M NaCl prior to transferring the DNA to a GeneScreen Plus (PerkinElmer) membrane overnight. An oligonucleotide complementary to the mitochondrial cytochrome *c* oxidase subunit II (*COII*) gene (see Table 1) was end labeled using $[\gamma\text{-}^{32}P]$ATP and T4 polynucleotide kinase. Following transfer, the DNA bound to the membrane was cross-linked and hybridized with the *COII* probe using ExpressHyb hybridization solution (Clontech) following the manufacturer's guidelines. The *COII* signal was quantitated using a FLA-5100 imaging system. The percentage of mtDNA remaining in infected cells is represented by the mtDNA/DNA ratio (*COII*/SYBR Gold) of a given sample normalized to the mtDNA/DNA ratio of mock-infected cells at four hours postinfection from at least three independent Southern blots.

Western blotting. Sample preparation for Western blotting was performed as previously described (16). Briefly, HeLa cells were transfected for 24 h and were lysed in radioimmunoprecipitation (RIPA) buffer. Alternatively, Vero cells were mock infected or infected for 4, 8, or 12 h, followed by lysis in IP lysis buffer. The membranes were blocked using 1:1 Odyssey blocking buffer (LI-COR)–Tris-buffered saline containing 5% Tween 20 (OBBT) and then incubated with one or more of the following primary antibodies: rabbit anti-FLAG antibody (Sigma), mouse anti-β-actin antibody (Sigma), mouse anti-ICP27 antibody (Virusys), mouse anti-glycoprotein C (gC) antibody (Virusys), rabbit anti-UL11 antibody (Rb 73 in reference 36), or mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Biodesign). The secondary antibodies used were goat anti-rabbit Alexa Fluor 680 antibody (Invitrogen) and donkey anti-mouse IRDye800 antibody (Rockland). All antibodies were diluted in OBBT. Proteins were visualized using an Odyssey Infrared Imaging System (LI-COR).

Immunofluorescence. HeLa cells grown on coverslips were transfected for 48 h and prepared for immunofluorescence as previously described (21). Cells were incubated with rabbit anti-FLAG (Sigma) and mouse anti-cytochrome *c* (BD Biosciences) primary antibodies followed by secondary antibodies goat anti-rabbit Alexa Fluor 555 antibody (Invitrogen) and goat anti-mouse Alexa Fluor 488 antibody (Invitrogen). The cells were washed with distilled H_2O and stained with 1 mg/ml 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 10 min. The stained cells were mounted on slides using Mowiol 4-88 (Sigma). Immunofluorescence microscopy was performed using a 63× objective and an ApoTome optical sectioning device (Zeiss).

Live-cell imaging of mtDNA depletion. HeLa cells grown in chambered coverglass slides (Nunc) were cotransfected for 48 h with 100 ng of pcDNA-Orange and 200 ng of the plasmids indicated below. After 48 h, the cells were stained with PicoGreen (3 μl/ml; Invitrogen) to visualize cytoplasmic mtDNA foci (37). Three separate experiments with at least 100 mOrange-positive cells per sample were performed. Live-cell fluorescence microscopy was performed using a 40× objective.

Viral growth curves. Vero cells were infected for 1 h at 37°C. The monolayers were then washed twice with phosphate-buffered saline, twice with acid wash (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0), and twice with DMEM, followed by incubation with complete Vero medium for the duration of the infection. Infected cells were harvested in the growth medium at the indicated times, frozen and thawed three times, and sonicated, and the debris was removed by centrifugation. The titer of each sample was determined on 6-5 cells.

Image processing and data analysis. Microscopy was performed with a Zeiss Axiovert 200M fluorescence microscope with AxioVision 4.5 software. Graphs were created using Microsoft Excel 2007. The final labeling and processing of figures was performed using Adobe Photoshop CS2. All statistical analysis was performed with two-tailed *t* tests with equal variance using Microsoft Excel 2007.

RESULTS

Eliminating translation of UL12.5 and UL12$_{M185}$ does not prevent mtDNA depletion. Our previous work examining mtDNA depletion by HSV-1 demonstrated that the viral protein UL12.5 is sufficient for mtDNA loss in transfected cells (9). However, the M127F virus that lacks the translational initiation codon of UL12.5 (22) still causes mtDNA depletion in the absence of UL12.5 expression (28). Our evidence pointed to an N-terminally truncated version of UL12.5, termed UL12$_{M185}$, as being responsible for mtDNA loss during M127F virus infection. We therefore asked whether eliminating the translation of both UL12.5 and UL12$_{M185}$ would prevent mtDNA loss.

We generated a UL12.5/UL12$_{M185}$ null virus (F/L) containing substitutions that convert the initiator methionine codons of UL12.5 (M127) and UL12$_{M185}$ (M185) to phenylalanine and leucine codons, respectively (Fig. 1). The presence of these mutations in the viral genome was confirmed through DNA sequencing (data not shown). Western blot analysis of infected cell extracts using a polyclonal UL12 antibody revealed that these mutations had no impact on the expression of full-length UL12 relative to its expression in wild-type or revertant (F/L Res) controls (Fig. 2A). In addition to full-length UL12, the wild-type and F/L Res extracts displayed several more-rapidly migrating species, including a prominent band corresponding to the mobility of UL12.5 (Fig. 2A). Although the F/L mutation is predicted to prevent translation of UL12.5, the band migrating at the mobility of UL12.5 was reduced but not eliminated in extracts prepared from cells infected with the F/L mutant. While the source of the residual signal is uncertain, it is possible that it arises through proteolysis of full-length UL12. Because the epitopes recognized by the polyclonal antiserum have not been mapped, it is not clear whether this species is truncated at the N terminus, the C terminus, or both ends.

To determine the effect of the F/L mutation on mtDNA depletion, we examined infected cell lysates for the presence of mtDNA by Southern blotting (Fig. 2B). As expected on the basis of previous work (9), wild-type KOS37 and KOS reduced the amount of mtDNA by more than 95% by 12 h postinfection ($P < 0.005$) (Fig. 2B), while the UL12, UL12.5, and UL12M$_{M185}$ null mutant AN1 had no effect (115% mtDNA relative to the amount in mock-infected cells; $P = 0.43$) (Fig. 2B). The F/L mutant displayed an intermediate phenotype where mtDNA levels were reduced by 71% compared to the amount in mock-infected cells at 12 h postinfection ($P = 0.02$) (Fig. 2B), which was significantly less than the degree of depletion mediated by KOS37 ($P = 0.029$) (Fig. 2B). Repairing the F/L mutation in F/L Res restored wild-type levels of mtDNA depletion, confirming that the defect displayed
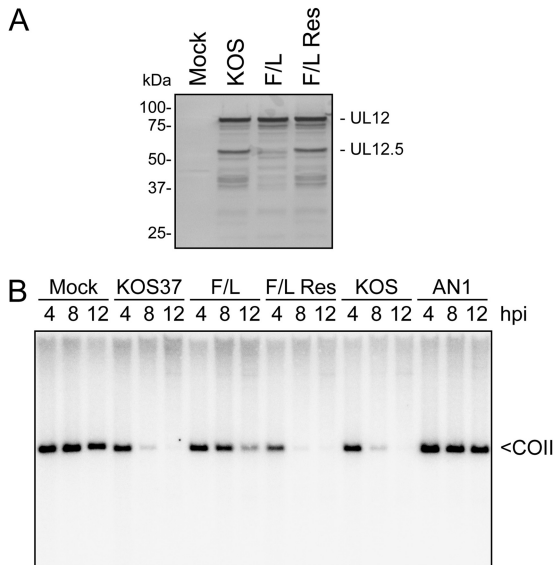
FIG 2 Preventing translation of UL12.5 and UL12$_{M185}$ impairs but does not abrogate mtDNA depletion. (A) Vero cells were mock infected or infected at a multiplicity of infection (MOI) of 10 with KOS, F/L, or F/L Res viruses for 8 h. Cell lysates were assessed for viral protein expression by immunoblotting using a polyclonal anti-UL12 antibody. (B) DNA from Vero cells mock infected or infected at an MOI of 10 with the indicated viruses was harvested 4, 8, or 12 h postinfection (hpi), and mtDNA was detected following Southern blotting using a radiolabeled probe directed against the mtDNA gene cytochrome *c* oxidase subunit II (*COII*).

by the F/L mutant is due to the UL12.5 mutations. These data indicate that inactivating the translation initiation codons of UL12.5 and UL12$_{M185}$ impairs but does not eliminate mtDNA depletion by HSV-1. Given that the AN1 null mutation abrogates mtDNA depletion, one or more products of the *UL12* locus appear to retain significant depleting activity in the F/L mutant. Perhaps low levels of intact UL12 are able to localize to mitochondria; alternatively, limited proteolysis of UL12 may give rise to low levels of a UL12.5-like product (Fig. 2A). Further studies are required to distinguish between these possibilities. In any case, these results demonstrate that the F/L virus is not suitable for examining the role of mtDNA depletion in HSV-1 replication.

UL12 orthologs from human beta- and gammaherpesviruses do not cause mtDNA depletion in transfected cells. The alkaline nuclease orthologs produced by HSV-2 (UL12), VZV (ORF48), HCMV (UL98), EBV (BGLF5), and KSHV (SOX) are all conserved in terms of their nuclease activities (7, 38–42), and a previous study has shown that UL98 can at least partially substitute for UL12 in promoting HSV-1 replication (43). However, it is not yet known if UL12 orthologs other than HSV-1 UL12.5 are capable of causing mtDNA depletion. To address this question, we created plasmids encoding C-terminally SPA-tagged versions of HSV-2 UL12, HSV-2 UL12$_{M117}$ (equivalent to HSV-1 UL12.5 [23]), VZV ORF48, HCMV UL98, EBV BGLF5, and KSHV SOX. All of the orthologs were expressed in transiently transfected HeLa cells, giving rise to products with the expected electrophoretic mobility (Fig. 3A); however, the levels of ORF48, BGLF5, and SOX were noticeably lower than the levels of the other orthologs. With the exception of HSV-2 UL12$_{M117}$, all of the UL12 orthologs localized predominantly to the nucleus and demonstrated no overlap with the mitochondrial protein cytochrome *c* (Fig. 3B). HSV-2
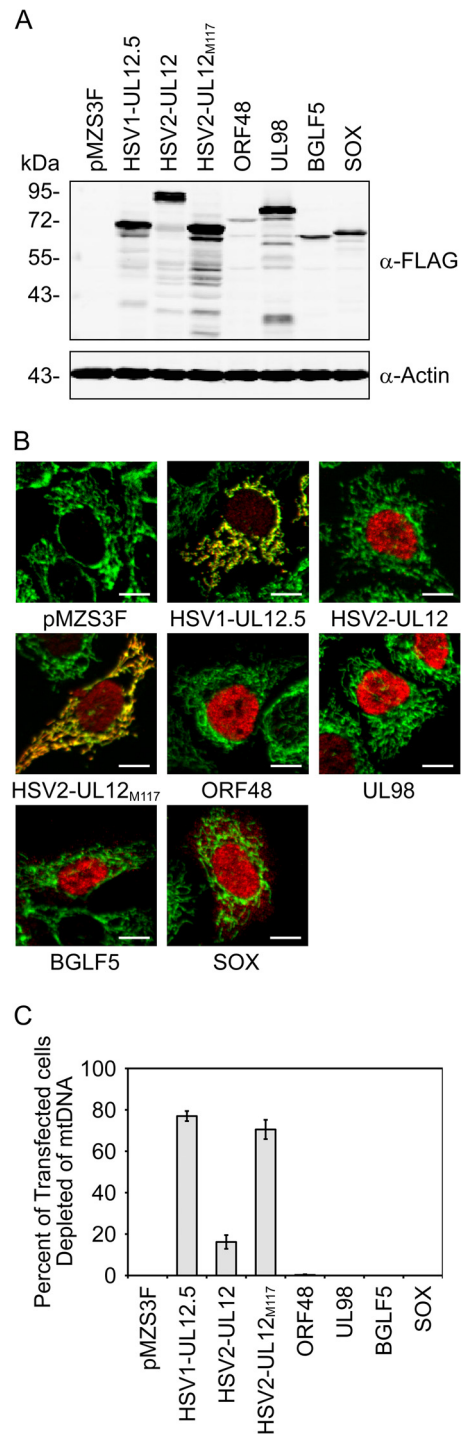
FIG 3 Ability of UL12 orthologs to deplete mtDNA. (A) The indicated plasmids were transfected into HeLa cells, and protein expression was visualized at 24 h posttransfection by immunoblotting with an anti-FLAG antibody. (B) Subcellular localization of the SPA-tagged proteins was examined in transfected HeLa cells by immunofluorescence. The mitochondrial protein cytochrome *c* is shown in green, and the SPA-tagged proteins (stained with anti-FLAG antibody) are shown in red. Colocalization with cytochrome *c* is indicated in yellow. For clarity, the DAPI channel has been omitted. Scale bars = 10 μm. (C) The presence or absence of mtDNA in transfected HeLa cells was scored by examining cytoplasmic PicoGreen staining in >100 randomly selected mOrange-positive cells. Data from three separate experiments were averaged, and standard errors are indicated.
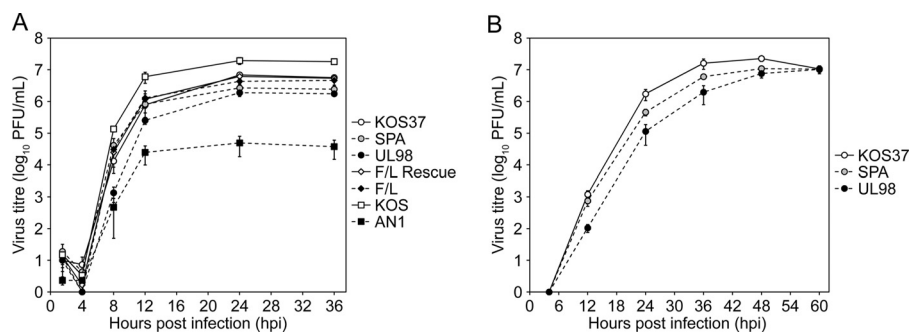
FIG 4 Replication of HSV-1 isolates used in this study. (A) Single-step growth curves were performed in Vero cells infected at a multiplicity of infection (MOI) of five with the indicated viruses and harvested at 1.5, 4, 8, 12, 24, and 36 h postinfection. (B) Multistep growth curves were performed in Vero cells infected at an MOI of 0.01 with the indicated viruses and harvested at 4, 12, 24, 36, 48, and 60 h postinfection. Determination of titers was performed on the UL12-complementing cell line 6-5 for all isolates, with the resulting titers plotted on logarithmic scales. KOS37-derived viruses are shown by circles and diamonds. KOS-derived viruses are shown by squares. Wild-type viruses are indicated with solid lines, and mutant viruses with dashed lines. Data are averaged from three independent experiments, and standard errors are indicated.

$UL12_{M117}$ localized to both the nucleus and mitochondria, similar to HSV-1 UL12.5 (9). In addition to strong nuclear localization, SOX and BGLF5 displayed diffuse cytoplasmic staining, consistent with the ability of these proteins to facilitate the turnover of cytoplasmic mRNAs (7, 8).

Next, we tested the ability of the various UL12 orthologs to deplete mtDNA in transiently transfected HeLa cells using a previously described live-cell imaging assay (9, 37). Consistent with its high degree of similarity to HSV-1 UL12.5, the HSV-2 $UL12_{M117}$ protein caused mtDNA depletion in the majority of transfected cells (Fig. 3C). We also observed a lower level of mtDNA depletion in cells expressing full-length HSV-2 UL12-SPA (Fig. 3C), likely due to the expression of HSV-2 $UL12_{M117}$ from its native promoter, as observed previously for HSV-1 *UL12* (16). In contrast, ORF48, UL98, BGLF5, and SOX did not detectably deplete mtDNA in this assay (Fig. 3C). Although it is currently unknown whether any of these genes produce N-terminally truncated proteins targeted to mitochondria, these data demonstrate that, aside from HSV-1 and HSV-2 *UL12*, none of the full-length orthologs produce proteins capable of depleting mtDNA in a transfection assay. This finding suggested that these orthologs might also be incapable of triggering mtDNA depletion during virus infection.

HCMV UL98 supports wild-type levels of HSV-1 replication. A previous study demonstrated that HCMV UL98 is able to at least partially complement the growth defect of the UL12-null HSV-1 AN1 virus (43). Given that UL98 also appeared to be unable to deplete mtDNA (Fig. 3C), we decided to generate an HSV-1 UL12 and UL12.5 null mutant that expresses UL98 from the *UL12* promoter. Using *en passant* mutagenesis, we disrupted the *UL12* and *UL12.5* ORFs by inserting *UL98* coding sequences to investigate the role of mtDNA depletion during infection. In this mutant virus, the *UL98-SPA* ORF was inserted in place of nucleotides 1 to 1152 of the *UL12* ORF (Fig. 1). The remaining 729 nucleotides at the 3′ end of the *UL12* ORF were retained to avoid disrupting the *UL11* promoter and coding sequence (Fig. 1), which overlap *UL12* in wild-type HSV-1 (23). To control for any effects of shifting the *UL11* gene downstream from *UL12*, we also created a virus which expresses UL12-SPA and UL12.5-SPA (KOS37 SPA) in a configuration similar to that of the KOS37 UL98-SPA genome (Fig. 1). The construction of this virus required that the 729-nucleotide sequence corresponding to the 3′ end of *UL12-SPA/UL12.5-SPA*

be recoded with silent mutations to allow UL12-SPA/UL12.5-SPA expression while avoiding sequence duplication and recombination with the downstream wild-type 729 nucleotides (containing the 5′ end of *UL11*). In these mutant viruses, UL98-SPA and UL12-SPA are under the control of the *UL12* promoter, while UL12.5-SPA is under the control of the *UL12.5* promoter.

Our next set of experiments directly examined whether the M127F/M185L mutations or the expression of UL98-SPA in lieu of UL12/UL12.5 had any impact on viral replication by performing growth analyses of all viruses used in this study. To perform single-step growth curves, Vero cells were infected with five PFU/cell and viral yields were assessed at various times postinfection (Fig. 4A). Consistent with previous observations (25, 33), the AN1 virus was significantly impaired in replication (ca. 400-fold-lower titers) compared to the KOS virus by 24 h postinfection (Fig. 4A). Wild-type KOS and KOS37 grew roughly comparably, although the KOS37 virus yielded slightly lower titers than did KOS virus (Fig. 4A). This minor difference in titers was also observed during the initial characterization of the KOS37 strain (30). The F/L, F/L Res, and KOS37 SPA viruses produced titers similar to those of the parental KOS37 throughout the 36-h time course (Fig. 4A). The replication of the KOS37 UL98 virus was delayed compared to that of KOS37 at early times, but the final titers were comparable to those of the parental virus (KOS37) and the control virus (KOS37 SPA) (Fig. 4A).

To determine whether the KOS37 UL98 virus displays a defect at low multiplicities of infection compared to the growth of the KOS37 and KOS37 SPA controls, we also infected Vero cells with 0.01 PFU/cell and performed a multistep growth analysis (Fig. 4B). Consistent with the data presented in Fig. 4A, the KOS37 UL98 virus exhibited somewhat reduced viral titers at early times postinfection relative to the titers produced following KOS37 and KOS37 SPA infection (Fig. 4B). Relative to the titers of the control KOS37 SPA virus, difference in viral titers were statistically significant only at the 36-h time point (P = 0.04). However, by 48 h postinfection the titers of the KOS37 UL98 and KOS37 SPA viruses were indistinguishable. The titers for KOS, KOS37, F/L, F/L Res, KOS37 SPA, and KOS37 UL98 viruses were all within one log unit throughout the time course (Fig. 4B and data not shown), and the replication-defective AN1 virus never achieved titers higher than 3.6×10^3 PFU/ml (data not shown).
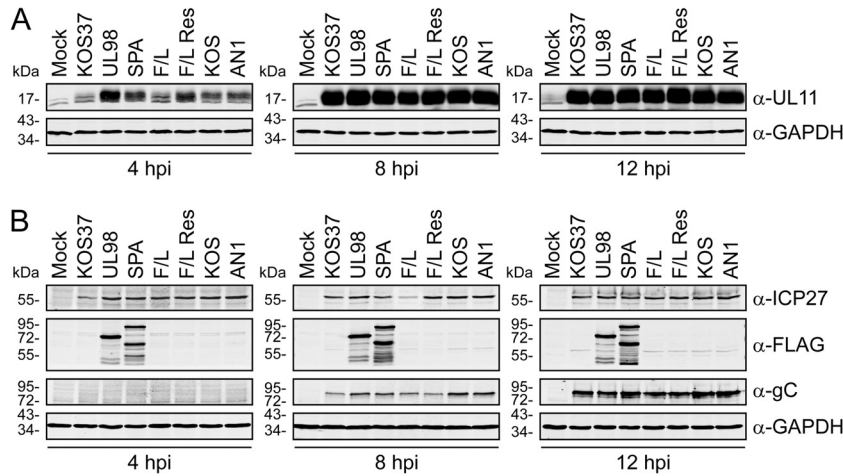
FIG 5 Viral protein expression is unaffected by mutations of the *UL12* gene. Vero cells were mock infected or infected at an MOI of 5 with the indicated viruses, and lysates for Western blotting were prepared at 4, 8, or 12 h postinfection (hpi). The expression of UL11 (A), ICP27 (B), glycoprotein C (gC) (B), and the FLAG-tagged proteins UL98-SPA, UL12-SPA, and UL12.5-SPA (B) is shown. GAPDH was included as a loading control.

Altogether, these observations are consistent with the results of complementation experiments involving UL98 during AN1 virus infection (43) and demonstrate that UL98 can restore viral replication to near wild-type levels in the absence of UL12 and UL12.5.

Viral protein expression. Since the KOS37 UL98 virus demonstrated a slight delay in viral replication, we examined whether this was correlated with delayed or abnormal viral protein expression. We first examined whether the expression of the early protein UL11 was disrupted due to the gene rearrangements in the KOS37 UL98 and KOS37 SPA viral genomes. Vero cells were infected with five PFU/cell of the various viruses for 4, 8, and 12 h, and the lysates were assessed for UL11 expression by Western blotting (Fig. 5A). At 4 h postinfection, UL11 expression was slightly higher in cells infected with KOS37 UL98, KOS37 SPA, and F/L Res viruses than in cells infected with the other viruses; however, these differences were no longer apparent at later time points (Fig. 5A). UL11 expression at or around 4 h postinfection is consistent with its known classification as a delayed early protein (44). An examination of additional viral proteins revealed, with the exception of some minor variations in protein expression (i.e., ICP27 in F/L infected cells at 8 h postinfection), that all mutant viruses expressed the immediate early protein ICP27 and the true late protein glycoprotein C (gC) at times consistent with their kinetic classes (Fig. 5B). We also observed clear expression of the FLAG-tagged protein UL98-SPA (ca. 75 kDa) or UL12-SPA (ca. 95 kDa) and UL12.5-SPA (ca. 70 kDa) from the KOS37 UL98 or KOS37 SPA viruses, respectively, at times associated with early protein expression (Fig. 5B). These data indicate that all of the mutant viruses expressed representative immediate early, early, and true late proteins with appropriate kinetics and that UL11 expression was not greatly affected by repositioning the *UL11* gene downstream from *UL12-SPA/UL12.5-SPA* or *UL98-SPA*. Thus, further experiments are required to determine the basis for the delayed replication of the KOS37 UL98 virus.

mtDNA depletion is severely impaired during infection with UL98-expressing HSV-1. We next tested the ability of the KOS37 UL98 and KOS37 SPA viruses to deplete mtDNA, using the Southern blot assay used for the experiments whose results are shown in Fig. 2B (Fig. 6). As expected, the KOS37 SPA virus reduced the

mtDNA content to levels similar to those observed with KOS and KOS37 by 12 h postinfection (KOS37 SPA, 2% remaining; KOS and KOS37, 5% remaining). In contrast, the KOS37 UL98 virus was severely impaired: 72% of the mtDNA remained at 12 h postinfection (Fig. 6), and this difference from the values for the mock-infected cells did not achieve statistical significance over the course of three independent experiments ($P = 0.24$). Altogether, these data indicate that replacing UL12, UL12.5, and $UL12_{M185}$ with HCMV UL98 severely impairs or inactivates the ability of HSV-1 to deplete mtDNA during productive infection and document that eliminating mtDNA is not required for efficient HSV-1 replication in cultured Vero cells (compare Fig. 4 and 6).

DISCUSSION

We have reached four major conclusions based on the data presented in this study. First, HSV-2 UL12 and $UL12_{M117}$ exhibit mtDNA-depleting activity similar to that of HSV-1 UL12 and UL12.5 (Fig. 3C). We consider it likely that the activity of the full-length HSV-2 UL12 construct stems at least in part from the production of $UL12_{M117}$ from its internal promoter, as observed with an HSV-1 UL12 expression vector (16). These data are strikingly similar to those which documented the involvement of UL12.5 in mtDNA depletion during HSV-1 infection (9, 28). In-
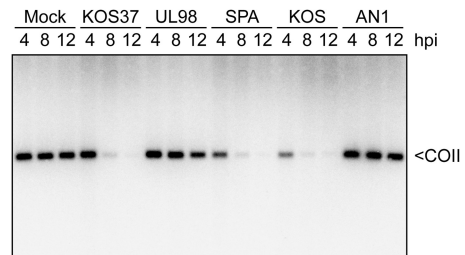
FIG 6 Expression of HCMV UL98 in lieu of HSV-1 UL12 severely impairs mtDNA depletion during HSV-1 infection. (A) Following mock infection or infection at an MOI of 10 with the indicated viruses, total DNA was isolated from Vero cells harvested at 4, 8, or 12 h postinfection (hpi). mtDNA was detected following Southern blot hybridization with a radiolabeled probe targeting the mtDNA gene cytochrome *c* oxidase subunit II (*COII*).

deed, the HSV-2 *UL12* locus gives rise to a 1.9-kb transcript with the potential to encode the $UL12_{M117}$ protein during infection (23). Due to the high degree of sequence conservation between HSV-1 and HSV-2, we propose that the HSV-2 $UL12_{M117}$ protein is responsible for mtDNA loss during HSV-2 infection (9) and suggest that it be designated UL12.5. Additional experiments are needed to confirm that UL12.5 is in fact produced during HSV-2 infection.

Our second conclusion is that, in contrast to HSV-1 and HSV-2, expression plasmids encoding full-length versions of the UL12 orthologs from VZV, HCMV, EBV, and KSHV are unable to cause mtDNA loss in transfected cells (Fig. 3C). Moreover, HCMV UL98 fails to deplete mtDNA when it is expressed from the HSV-1 genome during HSV-1 infection (Fig. 6). It is therefore possible that none of these *UL12* orthologs produce a mitochondrially targeted isoform analogous to HSV-1 and HSV-2 UL12.5, at least in our transfection assay. It would be interesting to determine whether any of these viruses produce such an isoform during productive infection. It is important to note that mtDNA depletion by HSV-1 UL12.5 does not require UL12.5 nuclease activity and involves the cellular mitochondrial nucleases ENDOG and EXOG (16). Therefore, even nuclease-deficient truncated isoforms may be sufficient to cause mtDNA depletion provided they are localized to mitochondria. These experiments would be especially informative for EBV because, as noted above, EBV depletes mtDNA during lytic infection, and Wiedmer et al. noted that the Zta protein is not sufficient for this effect in transfected cells, suggesting that other viral proteins may be involved (10). Taken in combination, the experiments outlined above will indicate how widespread mtDNA depletion is among human herpesviruses and will clarify the potential role of UL12 orthologs in this process for viruses other than HSV-1 and HSV-2.

Third, our data demonstrate that HCMV UL98 is able to efficiently substitute for HSV-1 UL12 during HSV-1 replication. Gao et al. have previously shown that UL98 can complement the growth defect of an HSV-1 UL12 null mutant (43); however, the efficiency of such complementation compared to that of HSV-1 UL12 was not determined. Our results confirm their observations and further demonstrate that UL98 supports the replication of UL12-null HSV-1 to essentially wild-type levels, albeit with a detectable delay at early times (Fig. 4). These data suggest that UL98 is able to interface with the HSV replication system almost as effectively as HSV-1 UL12. UL12 forms a complex with the HSV-1 single-stranded DNA binding protein ICP8 (24) and binds the host MRN complex (45). It will therefore be interesting to determine whether UL98 forms similar and functional complexes during infection with the KOS37 UL98 virus.

Finally, our results indicate that mtDNA depletion is not required for efficient HSV-1 replication in cultured Vero cells. mtDNA depletion was severely impaired in cells infected with the KOS37 UL98 virus (Fig. 6), and although a small decrease in mtDNA levels was observed at late times postinfection in some experiments, the difference was not statistically significant over repeated trials (Fig. 6). Since the KOS37 UL98 virus produced levels of progeny virus similar to those obtained with the control viruses (KOS37 and KOS37 SPA) as measured using single-step and multistep growth curves (Fig. 4), we can state with confidence that the elimination of mtDNA is not required for HSV-1 replication.

It is still unclear what benefit the loss of mtDNA serves during HSV-1 infection. Our observation that mtDNA depletion has little impact on virus replication in Vero cells suggests that it may serve a cell type-specific role or have a more obvious function in the intact host. It is clear from experiments with cells lacking mtDNA (rho^0) due to long-term ethidium bromide exposure that the absence of mtDNA ultimately compromises oxidative phosphorylation (46). Likewise, any loss of oxidative phosphorylation in HSV-infected cells likely occurs well after the initial depletion of mtDNA. Consistent with this view, Murata et al. demonstrated that HSV-1 and HSV-2 cause a decline in ATP production beginning around 12 h postinfection (47), after the viral replication cycle is largely complete. Thus, it seems unlikely that inhibition of oxidative phosphorylation plays a major role during productive infection. Recent work by Kramer and Enquist demonstrated that mitochondrial motility in neurons is inhibited by pseudorabies virus (PRV) and HSV-1 (48). Furthermore, this alteration in mitochondrial dynamics significantly enhanced PRV pathogenesis (48). However, the viral protein(s) required for the disruption of mitochondrial motility have yet to be identified. It will therefore be interesting to determine whether UL12.5 plays a role in HSV-induced inhibition of ATP production and/or mitochondrial motility or alters other mitochondrial properties, such as mitochondrial membrane potential, reactive oxygen species production, calcium homeostasis, the regulation of apoptosis or innate immune responses, or mitochondrial signaling pathways. In addition, it will be important to examine how HSV-1 UL12.5 alters mitochondrial biology in a variety of cell types, including cells which undergo nonproductive infection and therefore have the potential of surviving the initial infection. The KOS37 UL98 virus described here will be useful for such studies in cell culture, as well as for determining whether UL12.5 affects HSV-1 pathogenesis and/or latency in animal models.

ACKNOWLEDGMENTS

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