

# Nucleocytoplasmic Shuttling of Human Cytomegalovirus UL84 Is Essential for Virus Growth<sup>∇</sup>

Yang Gao,<sup>2</sup> Dominique Kagele,<sup>1</sup> Kate Smallenberg,<sup>3</sup> and Gregory S. Pari<sup>1\*</sup>

University of Nevada, Reno School of Medicine, Department of Microbiology and Immunology, Reno, Nevada 89557<sup>1</sup>; Nevada Cancer Institute, Drug Development Laboratory 6, One Breakthrough Way, Las Vegas, Nevada 89135<sup>2</sup>; and University of California, Berkeley, Department of Molecular and Cell Biology 142 Life Science Addition, Berkeley, California 94720<sup>3</sup>

Received 7 April 2010/Accepted 14 June 2010

**Human cytomegalovirus (HCMV) UL84 is a multifunctional protein that is the proposed initiator for lytic viral DNA synthesis. Recently it was shown that UL84 displays nucleocytoplasmic shuttling. The role of shuttling in lytic DNA replication and virus growth is unknown. We now show that expression of the nonshuttling UL84 mutant failed to complement oriLyt-dependent DNA replication in the transient assay under conditions where core replication and ancillary proteins were expressed under the control of their native promoters. However, constitutive expression of the core replication proteins, along with the nonshuttling UL84 mutant, resulted in efficient oriLyt amplification, suggesting that shuttling may contribute to the activity of one of the auxiliary replication proteins. A recombinant HCMV bacterial artificial chromosome plasmid (BACmid) expressing the nonshuttling UL84 mutant (NS84 BAC) was defective for production of infectious virus. Quantitative PCR showed that NS84 BAC had decreased accumulation of viral DNA in both cellular and supernatant samples. Analysis of the accumulation of select viral mRNAs showed no difference in total cellular mRNA accumulation for IE2, IRS1, TRS1, UL102, UL105, and UL75 in cells transfected with the NS84 BAC. However, examination of cytoplasmic RNA and subcellular localization of IRS1 revealed a decrease in IRS1 mRNA accumulation and displaced protein localization, strongly suggesting that UL84 facilitated the localization of IRS1 mRNA to the cytoplasm. RNA pulldown assays showed that UL84 interacted with IRS1 mRNA. These results indicate that nucleocytoplasmic shuttling is essential for virus growth and strongly suggest that UL84 is responsible for localization of at least one virus-encoded transcript, IRS1 mRNA.**

Human cytomegalovirus (HCMV) is widely distributed in the human population, with a majority of adults eventually becoming infected. The HCMV genome is a 229-kb linear double-stranded DNA (dsDNA) molecule that encodes approximately 200 genes (5, 34). During productive infection, HCMV gene expression occurs in three main phases: immediate-early, early, and late (11). Lytic viral DNA synthesis requires both the *cis*-acting origin of lytic DNA replication (oriLyt) (1, 2, 12, 21) and a set of *trans*-acting viral proteins (23, 24).

UL84 is essential for virus replication and growth (31, 35) and is one of the noncore proteins required for origin-dependent DNA replication in a transient replication assay (23, 28). UL84 interacts with oriLyt within the region that contains RNA/DNA hybrid and stem-loop structures and CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ) binding motifs (7, 14). The mechanism for initiation of HCMV DNA replication is proposed to be via a UL84 direct interaction with specific structures within oriLyt, and it is suggested that it could recruit other viral and/or cellular factors (4, 14). UL84 can interact directly with the HCMV regulatory protein IE2, which is the major transcription-activating protein of HCMV (29). UL84

also interacts and colocalizes with UL44, the polymerase accessory protein, which can form a replication complex with the other core proteins (10) (33).

HCMV UL84 contains amino acid motifs that are homologous with the proteins of DEX(H/D) box family (9). This class of proteins is involved in a broad range of activities. DEXH/D box proteins interact with the RNA component of ribonucleoproteins, unwind double-stranded RNA (dsRNA), shuttle RNA from the nucleus to the cytoplasm, can either up- or downregulate certain promoters, and can directly and actively displace stably bound proteins from RNA (6, 10).

Previous data showed that UL84 interacts with the cellular protein importin- $\alpha$ , and this binding domain comprises 282 amino acids within the UL84 protein (19). Further sequence analysis determined that UL84 contains a nuclear localization signal (NLS) in addition to two small, leucine-rich nuclear export signals (NES) (18). It was shown that mutation of amino acid residues L228A/L230A/L359A (LLL228/230/359AAA) completely abolished the nuclear export ability of UL84 and disrupted nucleocytoplasmic shuttling in a cell-based heterokaryon assay (18). To date, no known function is associated with this shuttling phenomenon.

To address the role of UL84 nucleocytoplasmic shuttling, transient transfection assays were performed in which we substituted a nonshuttling UL84 mutant for wild-type (wt) UL84. We show that UL84 shuttling can conditionally complement oriLyt-dependent DNA replication only when non-native constitutive expression of replication proteins is used

\* Corresponding author. Mailing address: Department of Microbiology, University of Nevada—Reno, Howard Bldg. 210, Reno, NV 89557. Phone: (775) 784-4824. Fax: (775) 327-2332. E-mail: gpari@medicine.nevada.edu.

<sup>∇</sup> Published ahead of print on 23 June 2010.

in the transient cotransfection-replication assay. However, expression of the required replication proteins and ancillary proteins from native promoters resulted in the inability of the UL84 nonshuttling mutant to complement oriLyt amplification. To further explore the role of shuttling in the context of the virus genome, an HCMV bacterial artificial chromosome plasmid (BACmid) expressing the nonshuttling UL84 mutant (NS84 BAC) was generated and transfected into human fibroblasts. No viral growth was detected, and real-time quantitative PCR (qPCR) analysis of cellular and supernatant viral DNA detected no increase in viral DNA for up to 8 days posttransfection (p.t.) for NS84 BAC, whereas a wild-type and a revertant HCMV BAC resulted in a marked increase in viral DNA accumulation. qPCR analysis of viral transcripts revealed no change in mRNA accumulation for all representative kinetic classes of genes in cells transfected with NS84 BAC or wild-type BAC at early time points. Subcellular localization of several viral proteins indicated that IE2 and IRS1 (inverted repeat short 1) proteins displayed an aberrant localization pattern in NS84 BAC-transfected cells. Analysis of cytoplasmic RNA showed a decrease in IRS1 mRNA accumulation. Further analysis determined that UL84 directly interacts with IRS1 mRNA, suggesting that a shuttling-defective mutant UL84 was responsible for transportation of this transcript. These data suggest that UL84 nucleocytoplasmic shuttling plays an important role in the viral life cycle and identifies at least one mRNA with which UL84 interacts.

#### MATERIALS AND METHODS

**Cells and antibodies.** Human foreskin fibroblast (HFF) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (Atlanta Biologicals). The UL84-specific monoclonal antibody MAb84 has been previously described (8). The IE2-specific antibody was purchased from Vancouver Biotech, Ltd. The anti-HCMV UL44 mouse monoclonal antibody was a gift from William Britt (University of Alabama School of Medicine). The UL83-specific antibody was purchased from Virusys (catalog number CA003-100). The anti-HCMV IRS1 mouse monoclonal antibody was provided by T. Shenk (Princeton University).

**Primers for BAC mutagenesis.** For the UL84 LL228/230AA mutation, the forward PCR primer, 5'-ATCTGCGCTCGGTAATTGACCAACAACACTGACGCGCATGGCCATCGTGCCTgtgacaattaatc-3', and the reverse primer, 5'-TAGCGGTAGCCGTTTCAACAGCGCGTGATGATACGGAGCGCGAAGAGATTtcagcaaaaggtcgattta-3', were used to amplify a *galk-kanamycin* (Kan) cassette (lowercase) flanked by BAC sequences (uppercase) from plasmid pGalK-Kan. The oligonucleotide includes the same flanking BAC sequence as the forward and reverse primers plus mutant sites (in boldface): CAACAAC TACGCGCATGGCCATCGTGCCTGCGCATCAGCCAATCTCTTCGCGCTCCGTATCATCACGCCGCTGTGAAACCGGCTA. For the UL84 L359A mutation, the forward PCR primer, 5'-CACTCCGCTTTGGACGATTGGAGTACACCGAACGAATCACC GCCGGACcctgtgacaattaatc-3', and the reverse primer, 5'-CACGTTGAAACGTAATATGCCGCTTTGGTATAGCGT GAGTGACGACGAGCGTtcagcaaaaggtcgattta-3', were used to amplify a *galk-kan* cassette (lowercase) flanked by BAC sequences (uppercase) from plasmid pGalK-Kan. The oligonucleotide includes both the same flanking BAC sequence as forward and reverse primers plus mutant sites (in boldface): GTCITTTGGA CGATTGGAGTAGACCGAACGAATCACC GCCGGACGCGAGCGCTGT CGTCACTACGCTATACCAAGACGGCATATTACGTTTC.

**Generation of recombinant BACmid expressing nonshuttling UL84.** *galk-kan*-based BAC mutagenesis was performed according to a protocol described previously (30). An overnight culture of HCMV BAC in SW102 cells was diluted 1:50 in Luria-Bertani (LB) medium with chloramphenicol selection and grown at 32°C in a shaking incubator to an optical density at 600 nm (OD<sub>600</sub>) of 0.6. The culture was then heat shocked at 42°C for exactly 15 min in a water bath. After 15 min, the culture was briefly cooled in an iced-water bath slurry and then pelleted in a tabletop centrifuge at 2,500 rpm at 4°C for 10 min. The supernatant

was poured off, and the pellet was resuspended in 12.5 ml of ice-cold double-distilled H<sub>2</sub>O (ddH<sub>2</sub>O) gently, and the samples were pelleted again. This step was repeated twice. After the second wash and centrifugation step, the supernatant was removed, and the pellet (~50 µl each) was kept on ice until electroporated with a gel-purified PCR fragment. An aliquot of 25 µl was used for each electroporation in a 0.1-cm cuvette (Life Technologies) at 330 µF and 400 V. After electroporation the bacteria were recovered in 700 µl of LB medium for 1 h in a 32°C shaking incubator. Transformants were selected at 32°C on LB agar plates containing chloramphenicol (30 µg/ml) and kanamycin (50 µg/ml). The next day several colonies were streaked on a MacConkey plate. Blood-red colonies were selected to do a miniprep, followed by Southern blot analysis. Once the correct *galk-kan* insertion was verified, the *galk* cassette was removed by the same method as previously described, except that a dsDNA oligonucleotide was used to replace the cassette, and bacteria were recovered for 4.5 h in a 32°C shaking incubator. After the recovery period, the bacteria were washed twice in 1× M9 salts and plated onto a counter-selection plate: M63, agar (15 g/liter), glycerol (0.2%), D-biotin (1 mg/liter), L-leucine (45 mg/liter), 2-deoxy-galactose (DOG; 0.2%), and chloramphenicol (30 µg/ml). After 4 days, colonies were picked, and a BAC miniprep was performed. Correct BAC mutants were confirmed by sequencing.

**Southern blot analysis.** HindIII-cleaved miniprep BAC DNA was visualized by ethidium bromide staining, denatured, and transferred to Zeta-Probe GT genomic-tested blotting membranes (Bio-Rad). DNA probes were radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP (PerkinElmer) with a Rediprime II random prime labeling system (GE Healthcare). Prehybridization was performed at 65°C for 1 h in hybridization buffer (7% sodium dodecyl sulfate [SDS], 10% polyethylene glycol, 1.5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaPO<sub>4</sub>, and 1 mM EDTA, pH 7.7]). DNA blots were hybridized with *galk*-radiolabeled probes in the same solution at 65°C overnight. The blots were washed twice for 15 min with 2× SSC-0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and twice for 30 min with 0.1× SSC-0.1% SDS at 65°C. The blots were imaged using a phosphorimager (GE Healthcare).

**Transient cotransfection-replication assay.** T-HFFs were cotransfected with plasmids encoding each of the HCMV core replication proteins under the control of the simian virus 40 (SV40) promoter, the IE2 expression construct pON2206, with or without the nonshuttling UL84 expression construct pHM2171NS UL84 and the UL84 expression plasmid pCMV 84TAG, pGEM-oriLyt, which contained the HCMV cloned oriLyt, using a calcium phosphate coprecipitation assay as described previously (32). Total cellular DNA was harvested 5 days posttransfection, cleaved with EcoRI and DpnI, and resolved on a 0.8% agarose gel. The DNA was transferred to a Zeta-Probe nylon membrane and hybridized with <sup>32</sup>P-labeled pGEM7z(-). The blots were imaged using a phosphorimager (GE Healthcare).

A replication assay with HCMV core replication proteins under the control of the native promoter was performed as described previously with the substitution of the nonshuttling UL84 expression plasmid pHM2171NS UL84 for the wild-type UL84 expression plasmid (24).

**Transfection of NS84 and wt BACs.** For each transfection, 7 × 10<sup>6</sup> actively dividing HFFs were trypsinized, suspended in 0.5 ml of Dulbecco's modified Eagle's medium plus 10% fetal bovine serum, and mixed with NS84 or wt BAC DNA, pcDNAp71tag (4), and sheared salmon sperm DNA in a 0.4-cm-gap cuvette. After electroporation, the cells were plated into a 60-mm dish with DMEM-10% fetal bovine serum. Culture medium was changed at 24 h posttransfection.

**Immunofluorescence assay.** Wild-type or NS84 HCMV BAC-transfected HFFs were plated on glass coverslips, fixed at 10 days posttransfection with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, and permeabilized with 0.2% Triton X-100 in PBS for 20 min at 4°C. Cells were rinsed with PBS, incubated with 10% goat serum for 20 min, and then rinsed again with PBS. Coverslips were incubated for 1 h at 37°C in a humidified tray with 2% goat serum and primary antibody. After incubation, the coverslips were washed in PBS three times for 5 min each and incubated with a 1:400 dilution of an Alexa Fluor 555-conjugated goat anti-mouse immunoglobulin antibody (Molecular Probes, Inc.) for 45 min at 37°C. Coverslips were washed in PBS three times and placed onto precleaned microscope slides. Images were captured using a confocal microscope with a 100× oil immersion objective. Subcellular localization of HCMV UL84 protein was visualized with an anti-HCMV UL84 MAB (1:200). Subcellular localization of HCMV IE2 protein was visualized with IE2 mouse monoclonal antibody G13-12E2 (1:300). The anti-HCMV UL44 mouse monoclonal antibody was used at a 1:200 dilution. Subcellular localization of HCMV UL83 protein was visualized with a 1:500 dilution of anti-HCMV UL83 mouse monoclonal antibody. The dilution of anti-HCMV IRS1 mouse monoclonal antibody was 1:10.

TABLE 1. Primers used for amplification of UL84-associated RNA

ORF	Forward primer (5'-3')	Reverse primer (5'-3')
IE2	GCAATTCTTTGAGGCTCCAC	CCGCAAGAAGAAGAGCAAAC
UL36 exon 2	GCGTACGAGGAACTCTTTGC	AGGAGTACCTGCACCCCTTT
UL37 exon 3	AGCGGAAATCTTCAAGCAAAA	CTGCGTGTCTTGAACGAGAG
IRS1	GGCAGTGGGAGTTCATGTTT	ATTCGTCGAGATCCAACCAG
TRS1	AACACCGTTTTCTCCAACG	TCAAGCTCTGGGACTGGACT
UL44	GGCTCGTTCATGAGATCGTT	CACCGGCCATCAAGTTTATC
UL105	CAAGAGGAGACCCACGACAT	TCTTGAGGTTCTTGGGATGG
UL102	AGTGGTCATAACGGCGGTAG	AGGCCTTGCCACATAGACG
UL84	GCTTACAGTCTTGCGGTTCC	GGGCTTATCTGGGTGTTTT
UL132	AAGGGCTCGAGGAACAAAAT	TGCTTCACATCGACTTCAGC
US28	CCGATTTGCTTTTCGTTTGT	AAGACGTACAGCAGCGGATT
UL75	AGGTAGTGAGTTCGCGGAGA	CCCAAACGGACAGTCAAAT

**Intracellular and supernatant viral DNA accumulation analysis by qPCR.** A 12-well dish was seeded with BAC-transfected HFFs. Cellular DNA was harvested at various times posttransfection (1, 2, 3, 4, 5, and 8 days) by applying DNA extraction buffer (2% SDS, 100 mM Tris-HCl [pH 8], 10 mM EDTA, and 50 mg of proteinase K/ml) directly to the culture dish. The cell lysates were incubated with proteinase K at 60°C for 1 h, followed by phenol-chloroform extraction and then chloroform extraction. The DNA was precipitated with 2 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate (NaAc). The precipitated DNA was resuspended in Tris-EDTA (TE) buffer, and total DNA was used in real-time PCR using TaqMan primers and a probe specific for the IE2 locus. Each experiment was performed in triplicate in an Eppendorf Realplex<sup>2</sup> detector. For supernatant viral DNA accumulation, culture medium was harvested at 3, 4, 5, and 8 days posttransfection. The supernatant, in addition to 2 ml of 25% sucrose, was centrifuged for 1.5 h at 100,000 × *g* using an SW41Ti rotor. The supernatant was discarded, and the virus pellet was resuspended in Hank's balanced salt solution (HBSS). The pelleted virus was DNase treated (Turbo DNase; Ambion) to remove any contaminating DNA, and viral DNA extraction and real-time PCR were performed as previously described.

**Total cellular RNA purification.** A six-well dish was seeded with wild-type and NS84 HCMV BAC-transfected HFFs. Total RNA was harvested at 2, 3, 4, and 6 days posttransfection and extracted with a PureLink total RNA purification system kit (Invitrogen) according to the manufacturer's instructions. Residual DNA contamination was eliminated by the use of Turbo DNA-free DNase (Ambion). cDNA was synthesized from 2 μg of total RNA in the presence of random hexamers, deoxynucleoside triphosphates, and Superscript III reverse transcriptase (Invitrogen). One microliter of the total cDNA was used in real-time PCR using TaqMan primers and a probe specific for the IE2, IRS1, TRS1 (immediate-early gene terminal repeat sequence 1), UL102, UL105, UL75, and UL44 genes. Each experiment was done in triplicate in an Eppendorf Realplex<sup>2</sup> detector.

**Cytoplasmic RNA isolation.** HFF cells transfected with BAC DNA (wt or NS84 BAC) were lysed at various times posttransfection in 400 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.5% Nonidet P-40, 1 mM dithiothreitol) with 4 μl of RNaseOUT (Invitrogen) on ice for 30 s. After centrifugation the supernatant was incubated with 10 μl of 20% SDS and 5 μl of proteinase K at 50°C for 60 min and then extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1), followed by ethanol precipitation of the RNA. The pellet was resuspended in 20 μl of H<sub>2</sub>O and treated with 1 μl of DNase at 37°C for 30 min. The final RNA was used for qPCR analysis. Results were normalized for differences in input BAC DNA measured at 24 h posttransfection.

**RNA coimmunoprecipitation.** HFF cells were infected with HCMV (multiplicity of infection [MOI] of 5), and at 5 days postinfection cells were lysed in a mammalian lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 2 mM MgCl) with protease and RNase inhibitors added. The lysate was centrifuged at 1,500 × *g* using a tabletop microcentrifuge to remove any unwanted cellular debris. Normal mouse immunoglobulin G conjugated to agarose beads (Santa Cruz Biotechnology) was added to the lysate, and the mixture was rotated at 4°C for 30 min. The normal mouse IgG beads were removed by centrifugation, and UL84 monoclonal antibody along with protein G-agarose beads (Santa Cruz Biotechnology) was added to the supernatant. The lysate plus antibody and protein G beads were incubated overnight at 4°C. The mixture was then centrifuged at 1,500 × *g*, and the beads were washed with 1 × Tris-buffered saline (TBS). RNA bound to the protein of interest was extracted/isolated using

TRIzol reagent according to the manufacturer's instructions. Isolated RNA was then reverse transcribed with Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol and amplified using specific primers for each of the identified HCMV genes (Table 1).

**Nested PCR.** RNA immunoprecipitations were performed as described above, with an additional immunoprecipitation using UL44-specific antibody (courtesy of William Britt). Reverse transcriptase PCR was performed as described above, using the following primer sets to amplify the IRS1 region between nucleotides (nt) 253 and 658 for the first round of PCR: forward primer, 5'-GAGCGCCT GTTGTGTCTCA-3', and reverse primer, 5'-CGTACACGAACACGCGCGG AAC-3'. Nested PCR was performed using product from the first round as the template, using the following primers to amplify the IRS1 region between nt 346 and 619: forward, 5'-CGTGTGCGCGCGGTTTTT-3', and reverse, 5'-CA GACCCTCGGGATGG-3'.

## RESULTS

**Conditional complementation of oriLyt-dependent DNA replication by a UL84 nonshuttling mutant.** As a first step to determine if nucleocytoplasmic shuttling activity contributes to the replication function of UL84, we used the cotransfection replication assay, substituting the nonshuttling UL84 mutant for the wild-type UL84 expression plasmid. In human fibroblasts, the original cotransfection replication assay used six core replication proteins (UL44, UL54, UL57, UL70, UL102, and UL105) and four additional HCMV-encoded loci (UL112-UL113 [UL112/113], UL84, IE2, and IRS1), all expressed from their native promoters (23, 24). Using these original assay conditions, nonshuttling UL84 (NS84) was unable to complement transient DNA replication (Fig. 1, lane 4), whereas wt UL84 was able to complement oriLyt replication (Fig. 1, lane 3). These data also demonstrate that the NS84 and wt UL84 expression plasmids are capable of complementing oriLyt-dependent DNA replication and that protein expression levels are adequate in this assay. The negative-control transfections, which omitted either the wt UL84 or IRS1 expression plasmid from the transfection mixture, also showed no oriLyt replication signal (Fig. 1, lanes 5 and 6). Transfection mixtures using plasmids encoding only the replication proteins UL44, UL54, UL57, UL70, UL102, and UL105 under the control of the SV40 early promoter (3) plus IE2 (under the control of its own promoter) showed that NS84 could complement oriLyt amplification (Fig. 1, lane 1). This modified assay, developed after the original assay, efficiently amplifies oriLyt and does not require the transfection of plasmids expressing IRS1 or UL112-UL113. These data suggest that the UL84 nucleocy-

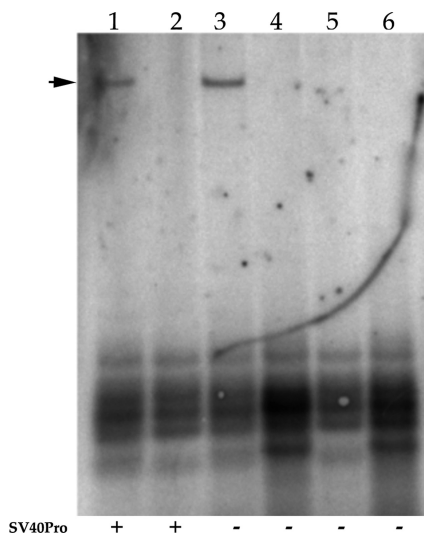


FIG. 1. Conditional requirement for nucleocytoplasmic shuttling of UL84 for oriLyt-dependent DNA replication. HFF cells were cotransfected with plasmids encoding all of the required core replication proteins under the control of the SV40 promoter or their native promoters plus an IE2 expression plasmid, oriLyt, and either a plasmid that expresses native UL84 or the nonshuttling mutant. Lanes: 1, SV40 promoter expression of replication proteins plus nonshuttling UL84 with IE2 and oriLyt; 2, SV40 promoter expression of replication proteins plus IE2 with oriLyt; 3, native promoter expression of replication proteins plus wt UL84 with IRS1, UL112/113, IE2, and oriLyt; 4, native promoter expression of replication proteins plus nonshuttling UL84 with IRS1, UL112/113, IE2, and oriLyt; 5, native promoter expression of replication proteins plus IRS1 with UL112/113, IE2, and oriLyt; 6, native promoter expression of replication proteins plus wt UL84 with UL112/113, IE2, and oriLyt. The arrow indicates replicated oriLyt.

toplasmic shuttling activity influences the expression or function of one or more auxiliary proteins involved in lytic DNA synthesis and that the shuttling component of UL84 is required when ancillary proteins are present. Lastly, the shuttling activity apparently does not directly contribute to the DNA replication function of UL84.

**Generation of a nonshuttling UL84 mutation in the HCMV BAC.** Since the NS84 subclone failed to complement oriLyt-dependent DNA replication under conditions where the replication proteins were expressed from their native promoters in the replication assay, we wanted to further investigate the function of UL84 nucleocytoplasmic shuttling in the context of the viral genome. We constructed a nonshuttling UL84 mutant BAC (NS84 BAC) using the GalK-Kan-based selection system. The amino acid residues 228 to 230 of the UL84 open reading frame (ORF) were replaced by the *galK-kan* resistant cassette, followed by the L228/230A mutant oligonucleotide replacement to generate a UL84 locus that contained the identical amino acid changes as those in the expression plasmid described above and as previously published (18). We then mutated UL84 L359A using the BAC template already containing the L228/230A change. The first step, insertion of the *galK-kan* cassette, was analyzed by HindIII cleavage, followed by Southern blot analysis using a *galK*-labeled probe. For the DNA gel, we observed a change in the cleavage pattern for the mutant

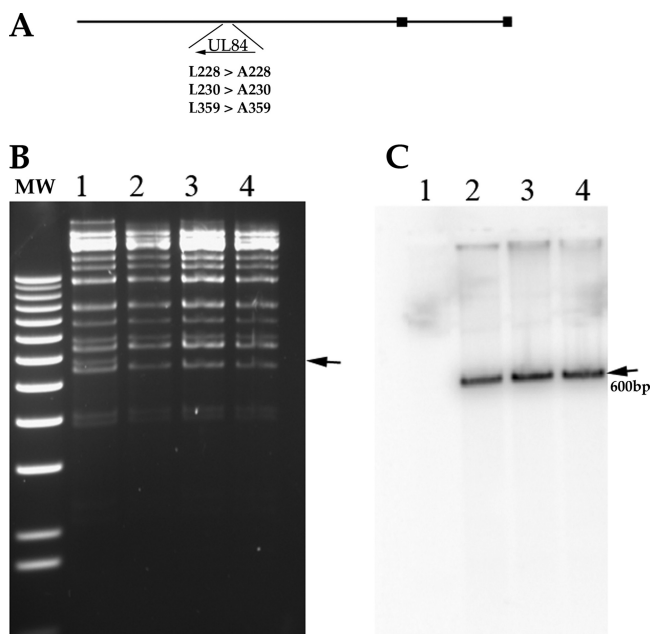


FIG. 2. Generation of HCMV nonshuttling UL84 recombinant BAC. (A) Schematic of HCMV genome showing the relative location of UL84 and the amino acid changes made to generate the HCMV NS84 BAC. (B) HindIII restriction enzyme cleavage of BAC DNA. Lanes: 1, wild-type HCMV BAC; 2 to 4 miniprep of three BAC colonies after recombination. The arrow shows the location of the wild-type band that is missing in the recombinant BAC DNA clones due to the insertion of the *galK-kan* cassette. (C) Southern blot analysis using the *galK* probe. The arrow indicates the location of the inserted *galK-kan* cassette in the recombinant BAC DNA. MW, molecular weight.

BAC compared to the wild-type BAC (Fig. 2A). As predicted, one band was detected in the Southern blot for the mutant UL84 BAC, indicating the presence of the *galK-kan* cassette, while no band was seen in blot of the wt HCMV BAC (Fig. 2B). Once we confirmed the correct *galK-kan* insertion in the recombinant BAC, this cassette was replaced with the NS84 mutant oligonucleotide, and several colonies containing the correct changes were confirmed by DNA sequence analysis.

**Nucleocytoplasmic shuttling of UL84 is required for virus growth.** Once the recombinant BAC was generated, we next wanted to determine if the NS84 BAC expressing nonshuttling UL84 could produce infectious virus and propagate DNA. We transfected HCMV wt BAC, NS84 BAC, and a revertant BAC along with a pp71 expression plasmid into HFFs. Transfection efficiencies were observed by the appearance of green cells due to the expression of green fluorescent protein (GFP) in the HCMV BAC genome. Photomicrographs of wild-type BAC, NS84 BAC, and revertant BAC were taken starting at 2 days posttransfection (p.t.). By 7 days p.t. both wt BAC and revertant BAC showed the appearance of large plaques typical of HCMV infection (Fig. 3). However, no distinct plaque formation was observed at 7 days p.t. for the NS84 BAC-transfected cells (Fig. 3). The NS84 BAC did develop small plaques at 13 days p.t. (Fig. 3). Slightly larger plaques developed at 27 days p.t. (Fig. 3); however, NS84 BAC never progressed

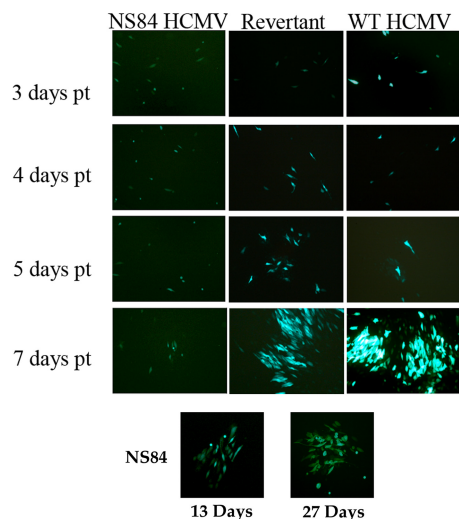


FIG. 3. HCMV NS84 BAC is defective for virus growth. HFF cells were transfected with NS84 BAC, revertant BAC, or wt BAC, and cells were allowed to incubate for up to 27 days posttransfection. Cells were visualized using a fluorescent microscope at 3, 4, 5, and 7 days posttransfection. For the NS84 BAC, images are also shown for 13 and 27 days posttransfection.

beyond this stage of plaque development even when BAC-transfected cells were incubated for up to 40 days (data not shown). Passage of NS84 BAC-transfected fibroblasts also did not result in plaque formation or measurable infectious virus from cells or supernatant (data not shown).

The BAC transfections were repeated, and results were confirmed several times with multiple BAC DNA maxipreps and different passages of HFFs to minimize the chance that negative results were caused by defects in the BAC DNA preparation or handling of the HFFs. These results indicate that nucleocytoplasmic shuttling is required for normal viral growth.

**NS84 BAC is defective for DNA synthesis.** Since NS84 BAC is defective in viral growth compared to the wild type, we wanted to determine whether the defect was due to DNA replication, viral packaging, or viral egress. To evaluate DNA accumulation in BAC-transfected cells, we used qPCR. HFFs were transfected with either wt BAC or NS84 BAC, and total cellular or supernatant DNA was harvested at various times points posttransfection. wt BAC viral DNA was shown to accumulate as much as 1,000-fold at 8 days p.t. (Fig. 4A). In contrast, relatively little viral DNA accumulation was observed in the supernatant of HFFs transfected with the NS84 BAC (Fig. 4A). Likewise, a similar increase in cellular DNA accumulation in wt BAC-transfected cells was observed from 3 days p.t. and reached 100-fold by 8 days p.t. (compared to the DNA measured at 2 days p.t.). However, cellular accumulation of viral DNA from NS84 BAC-transfected cells remained relatively unchanged days at 3 to 8 p.t. (Fig. 4B). Taken together, these results suggest that NS84 BAC is defective for DNA replication.

**Total mRNA accumulation is unchanged in NS84 BAC-transfected cells from representative kinetic classes of viral genes.** Since NS84 BAC was defective for viral growth and DNA replication, we next measured the mRNA transcript levels over a 6-day period in NS84 BAC- and wt BAC-transfected

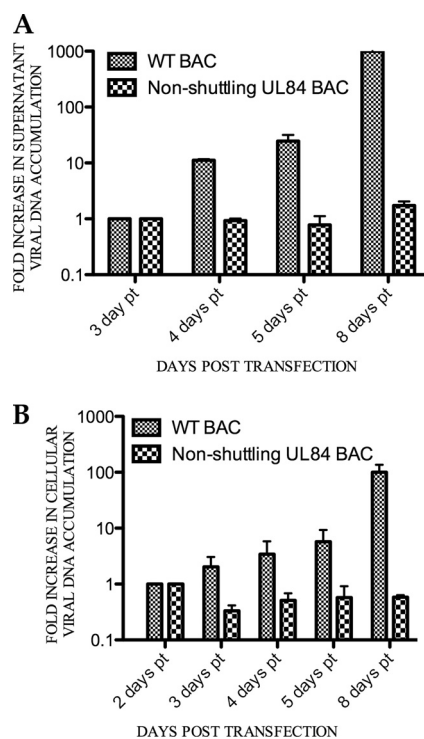


FIG. 4. Nonshuttling UL84 BAC is defective for DNA replication. (A) Evaluation of supernatant viral DNA accumulation from wt HCMV BAC and nonshuttling UL84 BAC-transfected cells. HFF cells were transfected with BAC DNA, supernatant virus was isolated, and viral DNA was extracted at 3, 4, 5, and 8 days posttransfection and assayed for viral DNA accumulation by real-time PCR. The data are presented as fold increase in viral DNA accumulation over the amount found at day 3 posttransfection. Error bars are standard deviations for three separate experiments. (B) Evaluation of intracellular viral DNA accumulation from wt HCMV BAC-transfected and NS84 BAC-transfected cells. The data are presented as fold increase in viral DNA accumulation over the amount of day 2 DNA.

HFFs. HFFs were transfected with wt or NS84 BAC, and total RNA was extracted at 2, 3, 4, or 6 days p.t. Relative mRNA levels were measured by using primers and probes for various kinetic classes of viral genes (Table 2).

IE2 mRNA levels in NS84 HCMV were similar to those observed for the wt HCMV for up to 4 days p.t. At 5 days p.t., concomitant with the spread of infectious virus (Fig. 5), IE2 levels for wt HCMV increased, whereas NS84 HCMV IE2 levels started to decrease slightly by approximately 2-fold (Fig. 5). This indicated that the increased accumulation of IE2 mRNA was due in part to the onset of DNA replication. For the mRNA accumulation of the IRS1, wild-type and NS84 HCMV displayed similar mRNA accumulation patterns as for IE2 except for a sharp increase (768-fold) in wt HCMV mRNA accumulation at 6 days p.t. (Fig. 5). The mRNA accumulation of the terminal repeat short 1 (TRS1) increased until 4 days p.t. in wt BAC-transfected cells. However, TRS1 mRNA accumulation did not significantly change after 2 days p.t. in NS84 BAC-transfected cells (Fig. 5).

We also measured mRNA accumulation for two early genes involved in DNA replication, UL102 (primase-associated factor) and UL105 (helicase), and one late gene (UL75). The

TABLE 2. Primers and probes used for real-time PCR

ORF	Primer direction or probe	Sequence (5'-3')
IE2	Forward	CTGTCAATCATGCCGGTATC
	Reverse Probe	GGGGAGAGGAGTGTAGTA CACGCTGACAACCCACT
IRS1	Forward	CGAGTCCTGGCGCCCGCT
	Reverse Probe	GTCCCCGAGGACCCAAT GCCTGGTTGGATCTCGA
TRS1	Forward	TCGTCGTCTCGTCTCAA
	Reverse Probe	GAAAACACTCGCGCTACA GGTGGTTCTGTGGGTC
UL102	Forward	AGAGTTATTGTCGCTCTCCA
	Reverse Probe	GCCGACGAAGAACAAGA CTTCCGTTCTCTCTCC
UL105	Forward	GAGGATTTTTCGTTCCAGTG
	Reverse Probe	GGGTTGTTATCTGTCGTTCTGTT TTTCGCTCCATCAGTC
UL75	Forward	ATCTGTCAATACCTCACGTT
	Reverse Probe	TGTGCGATCCCTTCCAGT CCACCCCAAACCACTCC

mRNA accumulation for UL102 and UL105 displayed a similar pattern to that observed for TRS1 (Fig. 5), except that there was a slight increase in accumulation in wt BAC-transfected cells. UL75 mRNA accumulation was also measured in wt and NS84 BAC-transfected HFF cells. The UL75 gene encodes the envelope glycoprotein H, which was used in our studies to demonstrate late gene kinetics (22, 36). The mRNA accumulation of UL75 from wt HCMV BAC-transfected cells increased in conjunction with the onset of viral DNA replication. However, UL75 mRNA accumulation from NS84 BAC-transfected cells increased after 3 days p.t. and had approximately the same mRNA fold increase as the wild type until 6 days p.t., at which point wt HCMV-infected cells showed a large increase in mRNA accumulation (Fig. 5). These results indicate that mRNA accumulation observed from cells transfected with NS84 BAC is similar to that observed in wt BAC-transfected cells at 3 to 4 days p.t.; however, marked differences between wild-type and NS84 viral transcription levels were observed by 6 days p.t. due to the onset of viral DNA synthesis in wt BAC-transfected cells.

**Evaluation of subcellular localizations of representative proteins in wt BAC-and NS84 BAC DNA-transfected cells.** Since there was apparently no defect in mRNA accumulation, we investigated the subcellular localization of several virus-encoded proteins by immunofluorescence assay. Cells were transfected with either wt HCMV BAC or NS84 BAC, and protein patterns were visualized using specific antibodies at 10 days p.t.

The localization patterns of UL84 were similar in both NS84 BAC- and wt HCMV BAC-transfected cells, indicating that the level and nuclear localization (mutant defect is in nuclear export) of UL84 were unaffected by the nonshuttling mutation (Fig. 6). The subcellular distribution of IE2 was slightly aberrant in the NS84 BAC-transfected cell samples. The IE2 subcellular pattern in wt HCMV BAC-transfected cells was typi-

cally nuclear, with the occurrence of globular structures (Fig. 6). However, the NS84 BAC-transfected sample showed a more distinct, smaller globular structure within the nucleus (Fig. 6). We observed no difference in the localization patterns of UL44 in cells transfected with NS84 and wt BAC (Fig. 6). The distribution of UL83 in cells transfected with NS84 BAC display the same pattern as that in cells transfected with wt HCMV BAC (Fig. 6). We also visualized the localization and occurrence of IRS1 protein in BAC-transfected cells. IRS1 was readily found in wt HCMV BAC-transfected cells; however, the IRS1 protein expression in NS84 BAC-transfected cells was not as prominent, despite the expression of other viral proteins in the same cells. Additionally, IRS1 was only weakly expressed in most NS84 BAC-transfected cells, whereas it was easily detected in wt HCMV BAC-transfected cells (Fig. 6). These experiments suggest that IRS1 (and IE2) subcellular localization and/or expression may be aberrant in cells transfected with the NS84 nonshuttling BACmid.

**Decrease in cytoplasmic IRS1 mRNA accumulation in non-shuttling NS84 BAC-infected cells.** The observed decrease in expression of IRS1 protein in cells transfected with NS84 BAC, coupled with the fact that total cellular mRNA levels were unchanged, prompted us to examine the accumulation of IRS1 mRNA in the cytoplasmic fraction. Since the NS84 is unable to shuttle from the nucleus to the cytoplasm, one plausible theory to explain this observation is that IRS1 mRNA is not being transported efficiently to the cytoplasm, and hence this leads to a decrease in protein expression. We transfected HFF cells with wt HCMV BAC or NS84 BAC DNA and isolated the cytoplasmic RNA fraction at 2 and 3 days p.t. qPCR was performed using primers and probes specific for several HCMV-encoded mRNAs. Of the HCMV-encoded transcripts evaluated, only IRS1 cytoplasmic mRNA displayed decreased accumulation compared to wt BAC-transfected cells (Fig. 7). Cytoplasmic mRNA accumulation for TRS1 was unchanged at 2 or 3 days posttransfection (Fig. 7). A slight decrease in IE2 cytoplasmic mRNA was also observed, whereas NS84 BAC-transfected cytoplasmic accumulations of UL105 and UL84 were similar to those observed in wt BAC-transfected cells (Fig. 7). Figure 7 also shows the corresponding total cellular RNA accumulation (bottom panel). Total cellular RNA accumulation is comparable to data shown above and indicates an increase in RNA for most genes at 3 days p.t. for the wt HCMV BACmid. These data strongly suggest that the shuttling activity of UL84 influences the transport of at least one virus-encoded mRNA.

**UL84 interacts with IRS1 mRNA.** The identification of a reduced accumulation of cytoplasmic IRS1 mRNA in cells transfected with a BACmid that expressed a nonshuttling version of UL84 suggested that UL84 influenced the cytoplasmic targeting of this transcript. We decided to investigate the possibility that UL84 interacts with RNA and specifically with IRS1 mRNA in infected cells. HFF cells were infected with HCMV, and an RNA immunoprecipitation was performed by pulling down UL84 protein along with any RNA species that were associated with the protein. As a control, we used an antibody specific for the K-bZIP protein for Kaposi's sarcoma-associated herpesvirus (KSHV) (15). The RNA component of the coimmunoprecipitation was extracted and used to generate cDNA. We used several

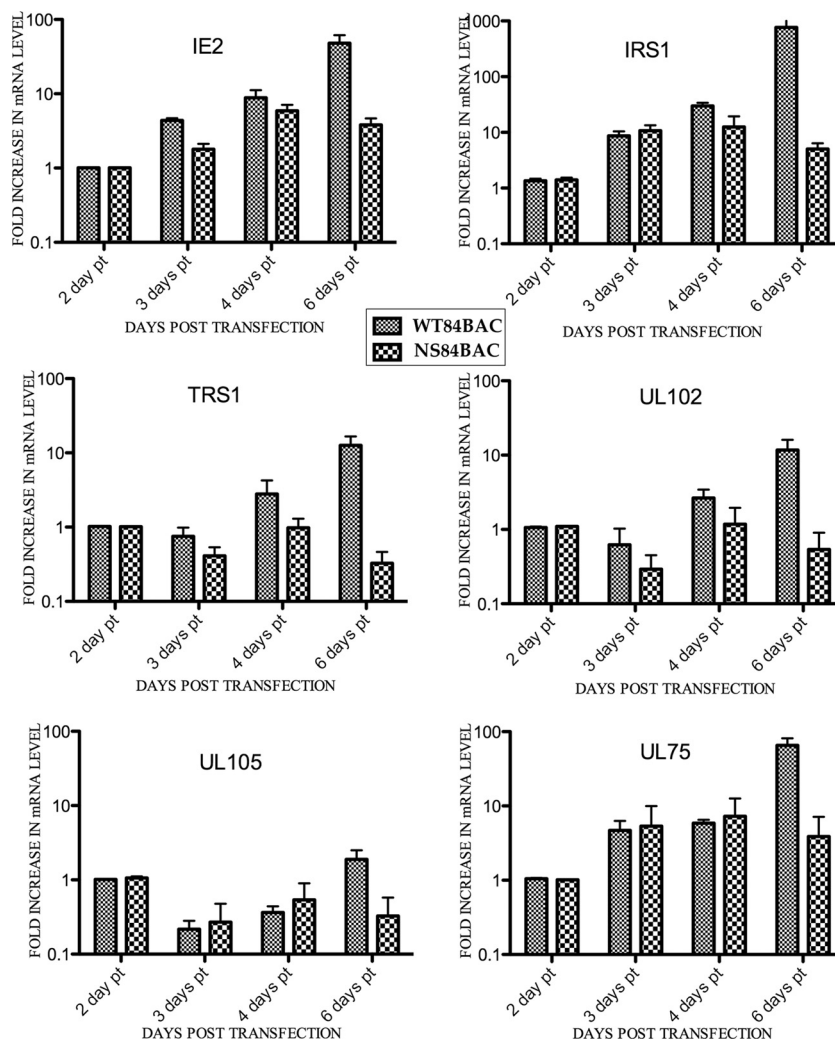


FIG. 5. Total cellular mRNA accumulation levels are similar in NS84 BAC- and wt HCMV BAC-infected cells for representative virus-encoded transcripts. HFF cells were transfected with either wt BAC or NS84 BAC DNA, and total cellular RNA was harvested at various days posttransfection and analyzed by qPCR. The data are presented as fold increase over mRNA accumulation measured on day 2 posttransfection. Error bars are the standard deviations from the averages of three separate experiments. Input DNA was measured at 20 h p.t. and found to be approximately equal for the wt BAC and NS84 BAC.

primer pairs specific for HCMV-encoded ORFs in an effort to identify RNA transcripts that interacted with UL84 in infected cells. Of the ORFs evaluated, a PCR-positive band was observed only for IRS1 mRNA (Fig. 8A, arrow). In order to confirm this result and control for nonspecific binding, we repeated the IRS1 immunoprecipitation along with an immunoprecipitation using an anti-UL44-specific antibody. In this confirmatory experiment we employed nested PCR for both immunoprecipitations using different first-round IRS1 PCR primers. The nested PCR results produced a stronger IRS1-specific band and no detectable band in the UL44 immunoprecipitated sample (Fig. 8B). Control samples, where the reverse transcriptase step was omitted, or a water control failed to produce a detectable PCR product (Fig. 8B). The results from these experiments suggest that UL84 can interact with IRS1 mRNA (directly or indirectly) and further suggest that the lack of cytoplasmic accumulation of at least one HCMV-encoded transcript may contrib-

ute to the observed phenotype for the nonshuttling mutant virus.

## DISCUSSION

The complexity of the regulation and initiation of HCMV lytic DNA replication is underscored by the multiple roles of UL84 within the virus life cycle. Since the discovery of the nucleocytoplasmic shuttling activity of UL84, it was postulated that this function could facilitate the localization of specific transcripts from the nuclear to cytoplasmic compartments. Our first step was to determine if the shuttling activity was required for lytic DNA replication. To this end, we used a well-characterized transient replication assay to show that nucleocytoplasmic shuttling of UL84 was required for oriLyt-dependent DNA replication under conditions where native promoters were used to express HCMV core replication proteins. This indicates that the shuttling activity of

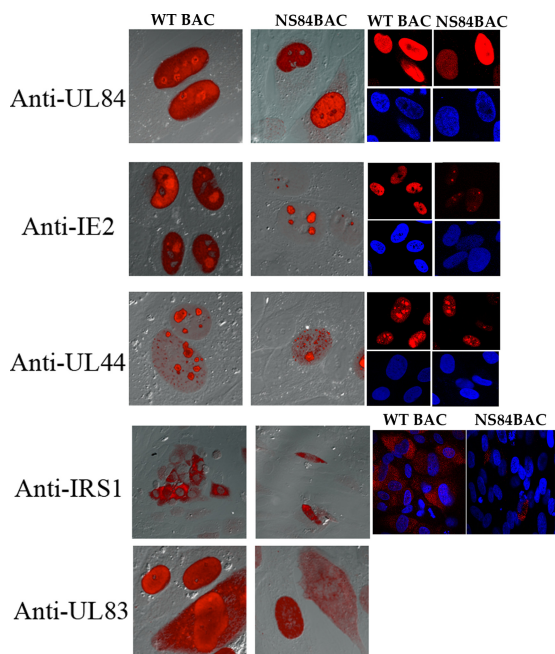


FIG. 6. IE2 and IRS1 display an aberrant subcellular localization pattern in NS84 BAC-transfected cells. HFF cells were transfected with wt or NS84 BAC DNA, and cells were visualized for the presence of UL84, IE2, UL44, UL83, and IRS1 by immunofluorescence using specific antibodies at 10 days posttransfection. Images are at a magnification of  $\times 40$  using Alexa Fluor 555-conjugated secondary antibodies. The presence of the indicated protein is shown in merged differential interference contrast (DIC) and bright-field images. DAPI (4',6'-diamidino-2-phenylindole)-stained cells are also shown to the right of DIC images.

UL84 does not directly contribute to DNA replication or affect the associated replication function of UL84. An adaptation of the original cotransfection assay, using strong constitutive promoters to express HCMV replication proteins, determined that UL84 shuttling was not required for HCMV origin-dependent DNA replication. The apparent defect in amplification of oriLyt when native promoters were used for protein expression suggested that the UL84 shuttling activity is required for the function of one or more of the ancillary replication proteins or the proper localization of one of the supplied proteins/RNA in the transfection mixture. Data indicate that nucleocytoplasmic shuttling of UL84 may be involved in the expression or proper localization of one or more additional factors: IRS1, UL112/113, and IE2. Previous results from transient transfection assays determined that UL84 was necessary for the formation of replication compartments in transfected cells (33). In addition, UL84 has been shown to colocalize with IE2 and UL44 in DNA replication compartments in the nucleus (33). UL44 and IE2 failed to form replication compartments when they were expressed from UL84-deleted BAC DNA-transfected cells (31).

In the context of the HCMV genome and using the mutant BACmid expressing the nonshuttling UL84 variant, the data presented here showed that the NS84 BAC was defective for viral growth and DNA synthesis. In NS84 BAC-

transfected cells, IE2 displayed an aberrant pattern within the nucleus, did not properly partition into replication compartments, and displayed punctate structures at 10 days p.t. These results suggest that the role of nucleocytoplasmic shuttling may regulate IE2 translation and localization. This aberrant pattern could be due to indirect effects of the shuttling activity of UL84 since we did not observe an interaction of IE2 mRNA with UL84. We did, however, observe a markedly different expression pattern for IRS1 in NS84 BAC-transfected cells, which likely contributed to the observed phenotype. Interestingly the NS84 mutant virus did form loose plaques at 13 days post-BAC DNA transfection. These plaques did not spread beyond a few infected cells, even when cultures were allowed to incubate for up to 27 days p.t. All attempts to harvest infectious virus from cells or supernatants failed even when transfected cells were passed up to three times (data not shown).

IRS1 along with TRS1 was shown to block the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2 $\alpha$ ) and the consequent shutoff of cellular protein synthesis that occurs during infection with vaccinia virus (VV) deleted of the double-stranded RNA binding protein gene E3L (VVDeltaE3L) (6). Recombinant viruses that lack both IRS1 and TRS1 have a severe replication defect (20). Although the study presented here shows that only IRS1 appears to be affected by the lack of shuttling by UL84, we do not exclude the possibility that other viral and/or cellular mRNAs are translocated by UL84, leading to the observed phenotype of the NS84 mutant virus. Nevertheless, we demonstrate for the first time that UL84 does interact with and shuttle a specific viral mRNA.

qPCR analysis of viral transcripts showed that in cells transfected with NS84 BAC, all representative kinetic classes of viral transcripts were produced at levels similar to those in wt BAC-transfected cells, except that the mRNA levels at 6 days p.t. had a significant fold increase in wt BAC-transfected cells due to DNA synthesis. These data suggest that nucleocytoplasmic shuttling of UL84 does not impact total cellular mRNA. Further experiments will be performed to check the cytoplasmic mRNA accumulation by RNase protection analysis.

Nucleocytoplasmic shuttling of UL84 may perform the same function as HIV-1 Rev protein. For example, Rev is required for the establishment of productive infection because it is essential for the cytoplasmic transport of unspliced and singly spliced viral transcripts (13). In herpes simplex virus type 1 (HSV-1), ICP27 shuttling plays an important role in the activation of HSV-1 late genes but is not involved in viral DNA replication (26). ICP27 is one of the best-characterized mRNA export factors that have been shown to favor viral RNA export and to retain cellular transcripts in the nucleus. ICP27 was shown to shuttle between the nucleus and cytoplasm through a leucine-rich nuclear export signal (16, 27) via a CRM1-dependent pathway (17). Interestingly, ICP27 and UL84 share many of the same functional characteristics associated with regulatory proteins. For example, UL84 shuttling is mediated through two leucine-rich nuclear export signals (18) and UL84 is an RNA binding protein (7). Another possibility is that UL84 shuttling may be facilitated via the interaction with a cellular



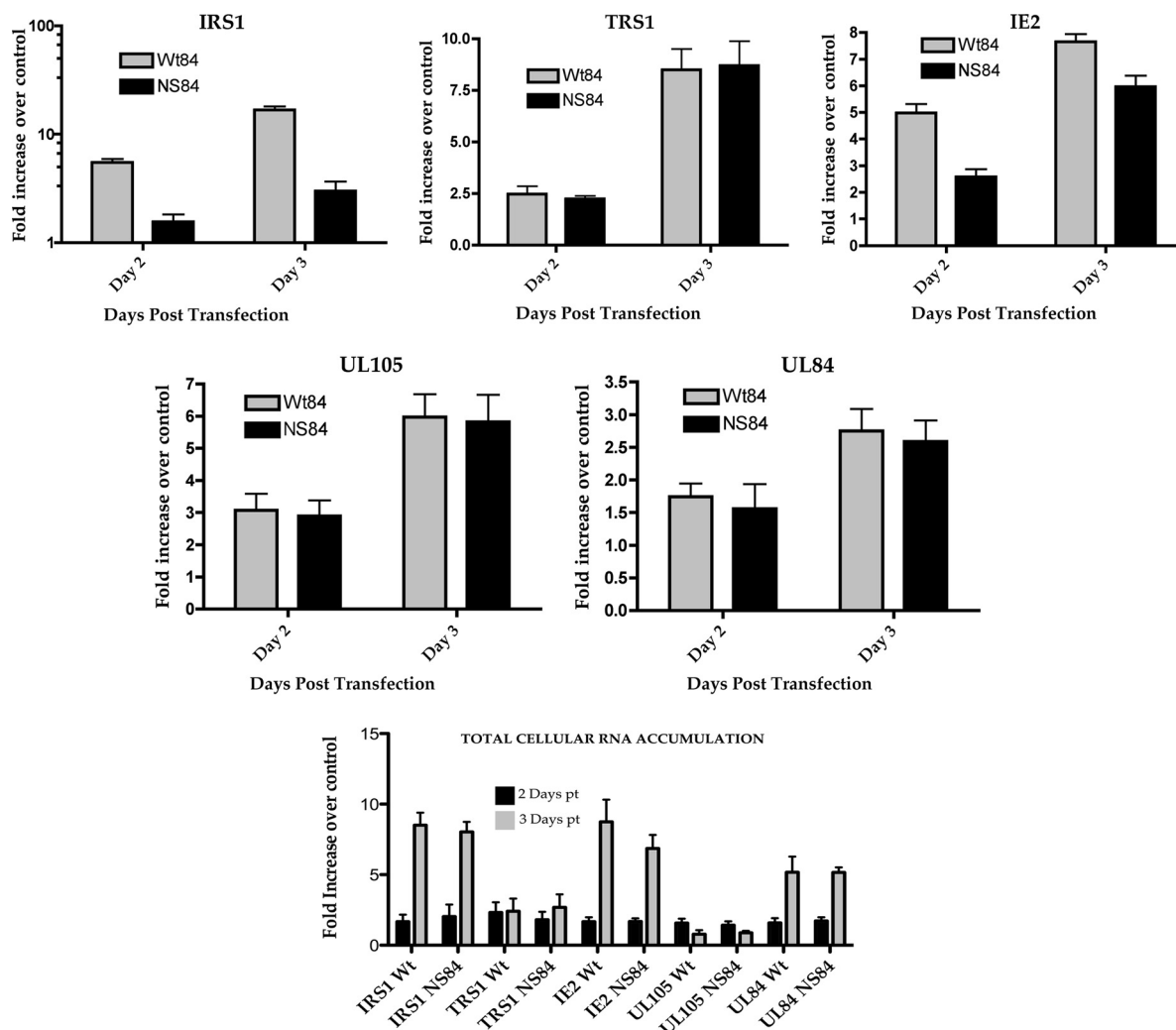


FIG. 7. Decreased accumulation of cytoplasmic IRS1 mRNA in NS84 BAC-transfected cells. HFF cells were transfected with wt or NS84 BAC DNA, and cytoplasmic RNA was harvested at 2 or 3 days posttransfection and analyzed by qPCR. Transfected BAC DNA was normalized, and the results shown are from three separate experiments (error bars). Total cellular RNA was evaluated using the same sample collected for cytoplasmic RNA evaluation. Data are reported as fold increase over the level at 1 day posttransfection.

protein and is not intrinsic to UL84 itself. We recently discovered that UL84 interacts with heterogeneous nuclear ribonucleoprotein K (hnRNP-K), a protein known to shuttle between the nucleus and the cytoplasm (data not shown). Hence, the transport and/or binding of mRNA by UL84 could be through a multiprotein complex.

The present study is based on a previous report by Lischka and colleagues in which they identified several amino acid motifs within UL84 that confer nucleocytoplasmic shuttling. Although we show that, in the transient assay, NS84 cannot complement oriLyt-dependent replication under conditions where ancillary proteins are present and are expressed from their native promoters, we have not demonstrated that our BACmid construct expresses a nonshuttling UL84 protein. Although we recognize this apparent weakness, we are confident that our study is valid since we have engineered the identical amino acid changes in the recombinant BACmid as were introduced into UL84 plasmids expressing the non-

shuttling version of the protein. Due to the low transfection efficiency of BACmid DNA and the lack of virus production in the NS84 BACmid, it is not possible to perform heterokaryon assays such as those done using infected cells (18).

Taking these results together, we conclude that novel nuclear export activity of pUL84 plays an important role in viral growth but does not contribute to DNA synthesis directly. Although additional studies are needed to elucidate how UL84 shuttling carries out these functions, the presently available evidence indicates that UL84 shuttling does impact IE2 distribution in the nucleus. It might be possible that UL84 shuttling is involved in IE2 translation because there is no significant difference in mRNA transcript levels of the IE2 gene between wt and NS84 BAC-transfected cells. It is clear, however, that IRS1 expression is directly influenced by UL84, and this is the first account of UL84 interacting with a virus-specific transcript. The exact mechanism(s) by which nucleocytoplasmic shuttling of UL84 reg-

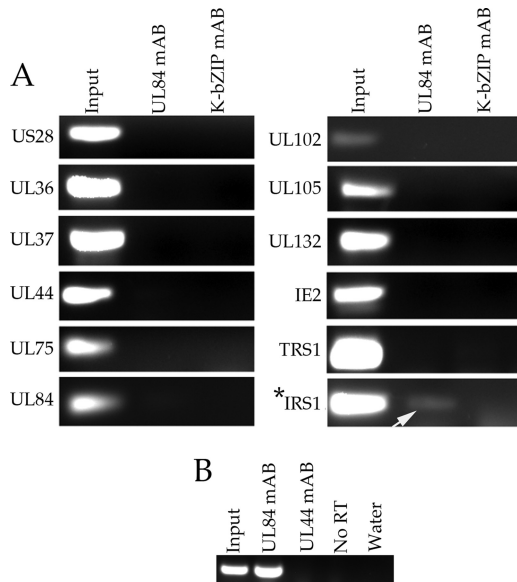


FIG. 8. UL84 interacts with IRS1 mRNA in infected cells. An RNA-protein pulldown assay was performed using HCMV-infected cells. Protein-associated RNA was used to generate cDNA, which was subsequently amplified using primers specific for various HCMV-encoded transcripts. For nested PCR, first-round PCR products were used as a template for a second round of PCR using primers that corresponded to sequences within the first-round PCR product. Control immunoprecipitations were done using anti-K-bZIP antibody (KSHV). The arrow indicates the presence of a positive PCR signal.

ulates viral and cellular genes during HCMV infection still needs to be elucidated.

#### ACKNOWLEDGMENTS

We thank Thomas Stamminger for the UL84 nonshuttling expression plasmids.

This work was supported by grant AI045096.

#### REFERENCES

- Anders, D. G., M. A. Kacica, G. Pari, and S. M. Punturieri. 1992. Boundaries and structure of human cytomegalovirus oriLyt, a complex origin for lytic-phase DNA replication. *J. Virol.* **66**:3373–3384.
- Anders, D. G., and S. M. Punturieri. 1991. Multicomponent origin of cytomegalovirus lytic-phase DNA replication. *J. Virol.* **65**:931–937.
- AuCoin, D. P., K. S. Colletti, S. A. Cei, I. Papouskova, M. Tarrant, and G. S. Pari. 2004. Amplification of the Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 lytic origin of DNA replication is dependent upon a *cis*-acting AT-rich region and an ORF50 response element and the *trans*-acting factors ORF50 (K-Rta) and K8 (K-bZIP). *Virology* **318**:542–555.
- Baldick, C. J., Jr., A. Marchini, C. E. Patterson, and T. Shenk. 1997. Human cytomegalovirus tegument protein pp71 (ppUL82) enhances the infectivity of viral DNA and accelerates the infectious cycle. *J. Virol.* **71**:4400–4408.
- Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, and T. Horsnell. 1990. Analysis of the coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* **154**:125–169.
- Child, S. J., M. Hakki, K. L. De Niro, and A. P. Geballe. 2004. Evasion of cellular antiviral responses by human cytomegalovirus *TRS1* and *IRS1*. *J. Virol.* **78**:197–205.
- Colletti, K. S., K. E. Smallenburg, Y. Xu, and G. S. Pari. 2007. Human cytomegalovirus UL84 interacts with an RNA stem-loop sequence found within the RNA/DNA hybrid region of *oriLyt*. *J. Virol.* **81**:7077–7085.
- Colletti, K. S., Y. Xu, S. A. Cei, M. Tarrant, and G. S. Pari. 2004. Human cytomegalovirus UL84 oligomerization and heterodimerization domains act as transdominant inhibitors of *oriLyt*-dependent DNA replication: evidence that IE2-UL84 and UL84-UL84 interactions are required for lytic DNA replication. *J. Virol.* **78**:9203–9214.
- Colletti, K. S., Y. Xu, I. Yamboliev, and G. S. Pari. 2005. Human cytomegalovirus UL84 is a phosphoprotein that exhibits UTPase activity and is a putative member of the DExD/H box family of proteins. *J. Biol. Chem.* **280**:11955–11960.
- Gao, Y., K. Colletti, and G. S. Pari. 2008. Identification of human cytomegalovirus UL84 virus- and cell-encoded binding partners by using proteomics analysis. *J. Virol.* **82**:96–104.
- Gilardi, K. V., A. Spinner, D. R. Canfield, C. R. Valverde, S. Hatcher, E. Larkin, J. Roberts, and M. McChesney. 2000. T-cell lymphoproliferative disorder in an aged rhesus macaque. *J. Am. Vet. Med. Assoc.* **217**:384–387.
- Hamzeh, F. M., P. S. Lietman, W. Gibson, and G. S. Hayward. 1990. Identification of the lytic origin of DNA replication in human cytomegalovirus by a novel approach utilizing ganciclovir-induced chain termination. *J. Virol.* **64**:6184–6195.
- Hope, T. J. 1999. The ins and outs of HIV Rev. *Arch. Biochem. Biophys.* **365**:186–191.
- Kagele, D., Y. Gao, K. Smallenburg, and G. S. Pari. 2009. Interaction of HCMV UL84 with C/EBP $\alpha$  transcription factor binding sites within *oriLyt* is essential for lytic DNA replication. *Virology* **392**:16–23.
- Kato-Noah, T., Y. Xu, C. C. Rossetto, K. Colletti, I. Papouskova, and G. S. Pari. 2007. Overexpression of the Kaposi's sarcoma-associated herpesvirus transactivator K-Rta can complement a K-bZIP deletion BACmid and yields an enhanced growth phenotype. *J. Virol.* **81**:13519–13532.
- Lengyel, J., C. Guy, V. Leong, S. Borge, and S. A. Rice. 2002. Mapping of functional regions in the amino-terminal portion of the herpes simplex virus ICP27 regulatory protein: importance of the leucine-rich nuclear export signal and RGG Box RNA-binding domain. *J. Virol.* **76**:11866–11879.
- Lengyel, J., A. K. Strain, K. D. Perkins, and S. A. Rice. 2006. ICP27-dependent resistance of herpes simplex virus type 1 to leptomycin B is associated with enhanced nuclear localization of ICP4 and ICP0. *Virology* **352**:368–379.
- Lischka, P., C. Rauh, R. Mueller, and T. Stamminger. 2006. Human cytomegalovirus UL84 protein contains two nuclear export signals and shuttles between the nucleus and the cytoplasm. *J. Virol.* **80**:10274–10280.
- Lischka, P., G. Sorg, M. Kann, M. Winkler, and T. Stamminger. 2003. A nonconventional nuclear localization signal within the UL84 protein of human cytomegalovirus mediates nuclear import via the importin  $\alpha/\beta$  pathway. *J. Virol.* **77**:3734–3748.
- Marshall, E. E., C. J. Bierle, W. Brune, and A. P. Geballe. 2009. Essential role for either *TRS1* or *IRS1* in human cytomegalovirus replication. *J. Virol.* **83**:4112–4120.
- Masse, M. J. O., S. Karlin, G. A. Schachtel, and E. S. Mocarski. 1992. Human cytomegalovirus origin of replication (*oriLyt*) resides within a highly complex repetitive region. *Proc. Natl. Acad. Sci.* **89**:5246–5250.
- McWatters, B. J., R. M. Stenberg, and J. A. Kerry. 2002. Characterization of the human cytomegalovirus UL75 (glycoprotein H) late gene promoter. *Virology* **303**:309–316.
- Pari, G. S., and D. G. Anders. 1993. Eleven loci encoding *trans*-acting factors are required for transient complementation of human cytomegalovirus *oriLyt*-dependent DNA replication. *J. Virol.* **67**:6979–6988.
- Pari, G. S., M. A. Kacica, and D. G. Anders. 1993. Open reading frames UL44, *IRS1*/*TRS1*, and UL36–38 are required for transient complementation of human cytomegalovirus *oriLyt*-dependent DNA synthesis. *J. Virol.* **67**:2575–2582.
- Reference deleted.
- Rice, S. A., and V. Lam. 1994. Amino acid substitution mutations in the herpes simplex virus ICP27 protein define an essential gene regulation function. *J. Virol.* **68**:823–833.
- Sandri-Goldin, R. M. 1998. ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. *Genes Dev.* **12**:868–879.
- Sarisky, R. T., and G. S. Hayward. 1996. Evidence that the UL84 gene product of human cytomegalovirus is essential for promoting *oriLyt*-dependent DNA replication and formation of replication compartments in cotransfection assays. *J. Virol.* **70**:7398–7413.
- Spector, D. J., and M. J. Tevethia. 1994. Protein-protein interactions between human cytomegalovirus IE2-5f80aa and pUL84 in lytically infected cells. *J. Virol.* **68**:7549–7553.
- Warming, S., N. Costantino, D. L. Court, N. A. Jenkins, and N. G. Copeland. 2005. Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res.* **33**:e36.
- Xu, Y., S. A. Cei, A. R. Huete, and G. S. Pari. 2004. Human cytomegalovirus UL84 insertion mutant defective for viral DNA synthesis and growth. *J. Virol.* **78**:10360–10369.
- Xu, Y., S. A. Cei, A. Rodriguez Huete, K. S. Colletti, and G. S. Pari. 2004. Human cytomegalovirus DNA replication requires transcriptional activation via an IE2- and UL84-responsive bidirectional promoter element within *oriLyt*. *J. Virol.* **78**:11664–11677.

33. **Xu, Y., K. S. Colletti, and G. S. Pari.** 2002. Human cytomegalovirus UL84 localizes to the cell nucleus via a nuclear localization signal and is a component of viral replication compartments. *J. Virol.* **76**:8931–8938.
34. **Yeung, K. C., C. M. Stoltzfus, and M. F. Stinski.** 1993. Mutations of the human cytomegalovirus immediate-early 2 protein defines regions and amino acid motifs important in transactivation of transcription from the HIV-1 LTR promoter. *Virology* **195**:786–792.
35. **Yu, D., M. C. Silva, and T. Shenk.** 2003. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. *Proc. Natl. Acad. Sci. U. S. A.* **100**:12396–12401.
36. **Yurochko, A. D., E. S. Hwang, L. Rasmussen, S. Keay, L. Pereira, and E. S. Huang.** 1997. The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF- $\kappa$ B during infection. *J. Virol.* **71**:5051–5059.