JOURNAL OF VIROLOGY, Apr. 2006, p. 3872–3883 Vol. 80, No. 8
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.8.3872–3883.2006


# Inhibition of Cellular DNA Synthesis by the Human Cytomegalovirus IE86 Protein Is Necessary for Efficient Virus Replication

Dustin T. Petrik,[1] Kimberly P. Schmitt,[2] and Mark F. Stinski[1,2]*

*Interdisciplinary Graduate Program in Molecular Biology[1] and Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242[2]*

Received 9 November 2005/Accepted 30 January 2006

**Human cytomegalovirus (HCMV) expresses several proteins that manipulate normal cellular functions, including cellular transcription, apoptosis, immune response, and cell cycle control. The IE2 gene, which is expressed from the HCMV major immediate-early (MIE) promoter, encodes the IE86 protein. IE86 is a multifunctional protein that is essential for viral replication. The functions of IE86 include transactivation of cellular and viral early genes, negative autoregulation of the MIE promoter, induction of cell cycle progression from $G_0/G_1$ to $G_1/S$, and arresting cell cycle progression at the $G_1/S$ transition in p53-positive human foreskin fibroblast (HFF) cells. Mutations were introduced into the IE2 gene in the context of the viral genome using bacterial artificial chromosomes (BACs). From these HCMV BACs, a recombinant virus (RV) with a single amino acid substitution in the IE86 protein was isolated that replicates slower and to lower titers than wild-type HCMV. HFF cells infected with the Q548R RV undergo cellular DNA synthesis and do not arrest at any point in the cell cycle. The Q548R RV is able to negatively autoregulate the MIE promoter, transactivate viral early genes, activate cellular E2F-responsive genes, and produce infectious virus. This is the first report of a viable recombinant HCMV that is unable to inhibit cellular DNA synthesis in infected HFF cells.**

Human cytomegalovirus (HCMV) is a member of the betaherpesvirus family. Like all herpesviruses, HCMV is an enveloped, double-stranded DNA virus. HCMV genes are expressed in a cascade of immediate-early (IE), early, and late genes. The IE genes are expressed in the absence of de novo protein synthesis and encode important functional proteins, including antiapoptotic and transactivation proteins. Early genes require the expression of IE proteins and encode proteins necessary for viral DNA replication. Following viral DNA replication, late genes are expressed and encode structural proteins for virion production.

Unlike the alphaherpesviruses, HCMV is a slow-replicating virus that replicates preferentially in terminally differentiated cells. Although the virus affects normal cellular physiology, including activation of signal transduction, transcription, cell cycle progression, and DNA synthesis (6, 22, 23, 25, 38, 42, 48, 57, 60), HCMV is unique because it is not associated with cell transformation like the gammaherpesviruses or other DNA tumor viruses.

The genome of HCMV is ~240,000 bp in size with at least 150 known open reading frames (ORFs) (9). A majority of the ORFs are nonessential for viral replication in cell culture. These nonessential ORFs likely encode proteins with redundant functions or proteins that may be required for replication in the human host. In addition to the nonessential ORFs, several ORFs are beneficial but not required for replication. However, approximately one-quarter, or 41 ORFs, are absolutely required for viral replication (59). UL122, an essential ORF, encodes the multifunctional IE86 protein which will be discussed in this paper.

* Corresponding author. Mailing address: 3-701 BSB, 51 Newton Rd., Department of Microbiology, University of Iowa, Iowa City, IA 52242. Phone: (319) 335-7792. Fax: (319) 335-9006. E-mail: mark-stinski@uiowa.edu.

The HCMV major immediate-early (MIE) locus consists of five exons, which are transcribed and alternatively spliced (51, 52). The MIE promoter regulates expression from the UL123 and UL122 ORFs, encoding the IE1 and IE2 genes, respectively. The IE72 protein, the predominant product of the IE1 transcript, is encoded by exons 2 and 3 spliced to exon 4. The IE86 protein, the predominant product of the IE2 transcript, is encoded by exons 2 and 3 spliced to exon 5. Translation of the IE1 and IE2 transcripts begins in exon 2; thus, the IE72 and IE86 proteins share the first 85 amino acids (51, 52).

While the IE72 protein is dispensable for HCMV replication at a high multiplicity of infection (MOI), the IE86 protein is essential for viral replication (13, 34, 37). IE86 is a 579-amino-acid protein with an approximate molecular mass of 86 kDa. The carboxyl-terminal portion of the protein, which is encoded by exon 5, is distinct from IE72 and contains essential functions of the IE86 protein. The protein is structurally and functionally complex. IE86 can be sumoylated and phosphorylated, contains two independent nuclear localization signals and an acidic activation domain, and forms homodimers (1, 16, 18, 31, 32, 41). IE86 interacts with a number of cellular and viral proteins, including basal transcription machinery TATA-binding protein (TBP), TFIIB, and TFIID; transcription factors CBP and CREB; histone acetyltransferase P/CAF; tumor suppressor proteins pRb and p53; and the viral UL84 protein (2, 5, 7, 11, 14, 15, 24, 26, 45, 47, 50).

The functions of the IE86 protein have been extensively studied using in vitro methods such as transient transfection of IE86 expression vectors, glutathione *S*-transferase pull-down, and gel electromobility shift assays using purified, recombinant IE86 protein or other assays not in the context of the entire viral genome. These experiments have revealed several functions of the viral protein. First, the IE86 protein binds to the *cis* repression sequence of the MIE promoter to negatively auto-

regulate expression of the IE1 and IE2 transcripts (8, 28, 40). Second, the IE86 protein transactivates viral early genes through its interactions with cellular basal transcription machinery (33). Third, the IE86 protein upregulates numerous cellular genes, including cyclin E and E2F-responsive genes (3, 48). Fourth, the IE86 protein promotes cell cycle progression in quiescent human foreskin fibroblast (HFF) cells from $G_0$ into $G_1$ (4, 56). Finally, the IE86 protein causes cell cycle arrest at the $G_1$/S transition in p53-positive cells (49).

Recently, several groups have investigated the unique region of the IE86 protein encoded by exon 5, using bacterial artificial chromosomes (BACs) which contain the HCMV genome. The use of HCMV BACs makes it possible to study the effect of mutations on the IE86 protein, in the context of the viral genome, even when the mutation results in a nonviable virus. While this approach can be useful to define regions of the viral protein that are important for viral replication, most of the mutations to date have been deletion mutants that affect multiple functions of the IE86 protein.

For this study, our goal was to study the effect of mutations within exon 5 of the IE2 gene, using HCMV BACs. However, random mutagenesis or alanine scanning strategies are not feasible in a region as large as exon 5 of IE2, so a more focused approach was needed. We instead introduced targeted mutations based on previously described mutations of the IE86 protein and amino acid sequence conservation between CMV species.

One such mutation was originally described in a nonfunctional IE86-expressing cell line (38). In this LXSN-IE86 cell line, several mutations were present, including the glutamine at amino acid position 548 mutated to an arginine (Q548R), which adds a positively charged residue in close proximity to the acidic activation domain (2, 38). Although the IE86 protein is known to arrest cell cycle progression, the LXSN-IE86 cells exhibited normal proliferation, suggesting that one of these mutated residues may be critical for this function. While most of the mutations were common strain variations or nonconserved residues, the Q548R mutation appeared to be unique. Therefore, we constructed a recombinant HCMV BAC containing the IE86 Q548R mutation and tested whether the Q548 residue is involved in the functions of the viral protein, including IE86 protein-mediated cell cycle arrest. The results and implications of those experiments are described below.

## MATERIALS AND METHODS

**Plasmids and BACs.** The pSVCS plasmid, containing the MIE promoter and UL123-UL121, was described previously (33). The Stratagene Quik-Change XL mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce point mutations into exon 5 of the IE2 gene in pSVCS, according to the manufacturer's instructions. The Q548R amino acid mutation and an EagI restriction enzyme site were introduced using the oligonucleotide 5′ G GCC TAC GCC GTC GGC CGG TTT GAG CAG CCC 3′ and its complementary oligonucleotide. The Q548A amino acid mutation and a KasI restriction enzyme site were introduced using the oligonucleotide 5′ G GCC TAC GCC GTG GGC GCC TTT GAG CAG CCC 3′ and its complementary oligonucleotide.

The pΔMCAT Δ−694/−583 plasmid, containing UL122-UL128 including the UL127/chloramphenicol acetyltransferase (CAT) reporter, was described previously (27, 30). The plasmid pGEM-T-kan/lacZ, kindly provided by T. Shenk (Princeton University, Princeton, NJ), was used to amplify the kanamycin resistance (Kan$^r$) gene using the primers 5′ AAA AAG GAT CCT GTG TCT CAA AAT CTC 3′ and 5′ AAA AAG GAT CCT CCT TCA ACT CAG CAA 3′. The Kan$^r$ gene was digested with the BamHI restriction enzyme to generate sticky ends and inserted between the UL127/CAT and UL128 ORFs of the pΔMCAT Δ−694/−583 plasmid at a BamHI site. The UL122-UL123 region of the pΔMCAT Δ−694/−583 plasmid was removed and replaced with UL121-UL123 of pSVCS, containing the mutations described above. The final shuttle vectors pΔMCAT Δ−694/−583 +Kan$^r$ +IE2 wild type (WT), pΔMCAT Δ−694/−583 +Kan$^r$ +IE2 Q548R, and pΔMCAT Δ−694/−583 +Kan$^r$ +IE2 Q548A were used for CAT assays and to construct recombinant BACs, described below.

The pΔMCAT Δ−694/−583 +Kan$^r$ +IE2 Q548R plasmid was further manipulated to generate a revertant (Rev) BAC. The plasmid pΩaacC4, kindly provided by T. Yahr (University of Iowa, Iowa), was digested with BamHI to isolate the gentamicin resistance (Gen$^r$) gene. The Kan$^r$ gene was removed from pΔMCAT Δ−694/−583 +Kan$^r$ +IE2 Q548R and replaced with the Gen$^r$ gene at the BamHI sites. The Q548R mutation in exon 5 of the IE2 gene was reverted to wild type using the oligonucleotide 5′ G GCC TAC GCC GTG GGG CAG TTT GAG CAG CCC 3′ and its complementary oligonucleotide. The final shuttle vector pΔMCAT Δ−694/−583 +Gen$^r$ +IE2 Rev was used for CAT assays and to construct a recombinant BAC, described below.

The HCMV Towne BAC DNA was kindly provided by F. Lui and described previously (9). Shuttle vectors were linearized by restriction digestion with NheI, and the UL121-UL128 fragment was gel purified. Recombinant BACs were generated using the linear UL121-UL128 fragment described above and homologous recombination with Towne BAC, in DY380 cells, as described previously (10). BAC DNA was isolated using the Nucleobond BAC Maxiprep kit (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions.

**Cell culture and recombinant viruses (RVs).** Primary HFFs were isolated and grown in Eagle's minimal essential medium (Mediatech, Herndon, VA) supplemented with 10% newborn calf serum (Sigma, St. Louis, MO) and penicillin (100 U/ml) and streptomycin (100 μg/ml). 293 cells were grown in Dulbecco's minimal essential medium (Mediatech), supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and penicillin (100 U/ml) and streptomycin (100 μg/ml).

Recombinant viruses were isolated, propagated, and maintained as described previously (21). Briefly, HFF cells were transfected with 1 or 3 μg of each recombinant BAC and 1 μg of the plasmid pSVpp71 using the calcium phosphate precipitation method (12). Extracellular fluid was harvested 5 to 7 days after 100% cytopathic effect (CPE) was seen and stored at −80°C in 50% newborn calf serum or passaged onto fresh HFF cells. Virus titers were determined by plaque assay, using equal viral DNA input from stored virus stocks, as described previously (20, 21).

**Multiplex real-time PCR analysis.** HCMV gB DNA was detected and quantified using multiplex real-time PCR for viral DNA input and viral DNA replication assays. HFF cells were infected, in triplicate, with various dilutions of stored recombinant virus for viral DNA input assay or transfected, in triplicate, with 3 μg of recombinant BAC DNA for viral DNA replication assay. Whole-cell DNA was harvested as described previously (19) at 4 h postinfection (hpi) for viral DNA input assay or at 1, 5, 9, 13, 21, and 28 days posttransfection for viral DNA replication assay. Multiplex real-time PCR was performed using 0.5 μg DNA in a final volume of 25 μl of the Platinum PCR Supermix-UDG cocktail (Invitrogen, Carlsbad, CA). HCMV gB primers and 6-carboxyfluorescein (FAM)–6-carboxytetramethylrhodamine (TAMRA) probe, described previously (19), and cellular 18S rRNA primers and VIC-TAMRA probe (Applied Biosystems, Foster City, CA) were used simultaneously. Thermal cycling conditions were, and relative quantification of gB DNA was performed, as described previously (35).

HCMV RNA was detected and quantified using multiplex real-time PCR for MIE autoregulation and viral early gene transactivation assays. HFF cells were transfected, in triplicate, with 5 μg of recombinant BAC DNA for MIE autoregulation assay or infected, in triplicate, with various dilutions of recombinant virus for viral early gene transactivation assays. Whole-cell RNA was harvested using TRI reagent (Invitrogen), treated with RNase-free DNase, and converted to cDNA as described previously (20). Multiplex real-time PCR was performed using 2 μl undiluted cDNA, or RNA lacking reverse transcriptase, in a final volume of 25 μl of the Platinum PCR Supermix-UDG cocktail (Invitrogen). Primers and FAM-TAMRA probes for HCMV MIE and TRS1 IE genes were described previously (35, 58). HCMV UL44 RNA was detected using primers 5′ TTT TCT CAC CGA GGA ACC TTT C 3′ and 5′ CCG CTG TTC CCG ACG TAA T 3′ and probe 5′ FAM-AGC GTG GCG ATC CCT TCG ACA AA-T AMRA 3′. HCMV UL54 RNA was detected using primers 5′ TTG CGG GTT CGG TGG TTA 3′ and 5′ CGG CCA TAG TGT TGA GCT TAT AGT T 3′ and probe 5′ FAM-TAC CCT GTA TGC ATG GCC AAG ACT AAC TCG-T AMRA 3′. HCMV IRL7 RNA was detected using primers 5′ GCC GAG AGA ATG CCA GTA AGA 3′ and 5′ GGT TAT TCG CTG GTT CGT TCT AA 3′ and probe 5′ FAM-CGA CCA TCG CAG ACA CAG TAC GAT ACT CA-

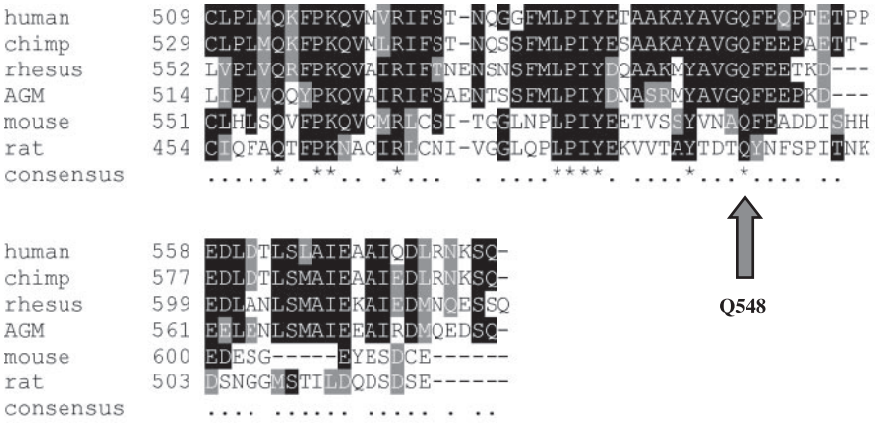


FIG. 1. Multiple sequence alignment of the CMV IE86 protein homologs. Amino acid sequences for the proteins from human CMV Towne strain IE2 (AAR31449), chimpanzee CMV IE2 (NP612745), rhesus CMV IE2 (AAB00488), African green monkey (AGM) CMV IE2 (AAB16881), mouse CMV IE3 (AAA74505), and rat CMV IE2 (AAB92266) were aligned using MultAlin. Multiple sequence alignments are displayed using BoxShade. Identical residues appear shaded in black, while similar residues appear shaded in gray. In the consensus sequence, a star indicates a residue that is identical in all aligned sequences, while a dot indicates a residue that is conserved in at least half of the aligned sequences. The numbers appearing between the species and the amino acid sequence represent the amino acid position for that particular species. A hyphen designates a gap in the sequence that was inserted for optimal alignment.

TAMRA 3′. HCMV UL44, UL54, and IRL7 primers and probes were designed and tested by Lian-Fai Yee (University of Iowa, Iowa). Thermal cycling conditions were, and relative quantification of HCMV RNA was performed, as described previously (35) Threshold cycle values of samples not treated with reverse transcriptase did not differ appreciably from those of baseline.

**Western blot analysis.** HFF or 293 cells were transfected with 5 μg of recombinant BAC DNA using the calcium phosphate precipitation method. Western blot analysis of viral proteins was performed as described previously (38, 39). Briefly, cell lysates were harvested at 24 h posttransfection and fractionated on a 9% polyacrylamide-sodium dodecyl sulfate gel. Proteins were transferred to a polyvinylidene difluoride membrane, and Western blotting was performed using primary mouse monoclonal antibody 810 (Chemicon), to detect MIE proteins, or primary mouse monoclonal antibody E7, to detect cellular β-tubulin. Proteins were detected using secondary horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) and Pierce SuperSignal West Pico chemiluminescence detection reagent (Pierce Biotechnology, Rockford, IL).

**CAT assay.** Viral activation of the early UL127/CAT reporter gene was determined by CAT assay, as described previously (30). Briefly, HFF or 293 cells were transfected, in triplicate, with 3 μg IE86/CAT shuttle vector and 2 μg β-galactosidase (β-Gal) reporter plasmid. Total protein was harvested at 4 days posttransfection. CAT assays were performed, and the percentage of acetylated [$C^{14}$]chloramphenicol (Perkin-Elmer Life Sciences, Boston, MA) was determined by image analysis. Bradford assays were performed to normalize the CAT activity per microgram of protein. β-Gal assays were performed to normalize the CAT activity for transfection efficiency.

**BrdU incorporation and immunofluorescent microscopy.** Cellular bromodeoxyuridine (BrdU) incorporation was visualized by immunofluorescent microscopy, as described previously (49). Briefly, confluent contact-inhibited HFF cells were serum starved for 48 h to synchronize in $G_0$. Cells were infected for 6 h in medium containing 0.05% serum with Towne, Q548A, or high-titer Q548R RV to preexpress IE proteins. Cells were then split onto glass coverslips for release from contact inhibition and grown for 42 h in medium containing 10% serum, to promote cell cycle progression, plus 10 μM BrdU, to detect DNA synthesis. Cells were fixed and permeabilized in a cold 1:1 mixture of methanol and acetone for 10 min. Permeabilized cells were stained with primary rabbit antibody 6655 (a gift from J. Nelson, Oregon Health Sciences University) and secondary goat anti-rabbit IgG conjugated with Alexa 488 (Molecular Probes, Eugene, OR) to detect IE86 protein, with primary mouse antibody to BrdU (Amersham Biosciences) and secondary goat anti-mouse IgG conjugated with Alexa 568 (Molecular Probes) to detect DNA synthesis, and counterstained with DAPI (4′,6′-diamidino-2-phenylindole). Cells were visualized by fluorescent microscopy with an Olympus BX-51 light microscope.

**Flow cytometry analysis.** Cell cycle analysis of infected cells by fluorescence-activated cell sorting (FACS) was performed as described previously (38). Briefly, HFF cells were grown to 50% confluence and then serum starved for 48 h to synchronize in $G_0$. Cells were infected for 6 h in medium containing 0.05% serum with Towne, Q548A, or high-titer Q548R RV to preexpress IE proteins and then grown in medium containing 10% serum for 42 h to promote cell cycle progression. Cells were trypsinized, pelleted, and permeabilized in cold 70% methanol for 1 h. Permeabilized cells were stained with primary rabbit antibody 6655 and secondary goat anti-rabbit IgG conjugated with Alexa 488 to detect IE86 protein, counterstained with DAPI, and subjected to FACS analysis. IE86-positive infected cells were gated and assayed for DNA content. The cell cycle profile of infected cells was plotted, and the percentage of cells in each cell cycle compartment was calculated using the ModFit software program.

**Northern blot analysis.** Northern blot analysis of RNA from infected cells was performed as described previously (48). Briefly, confluent contact-inhibited HFF cells were infected, in triplicate, with Towne, Q548A, or high-titer Q548R RV. Cytoplasmic RNA was harvested at 48 hpi and treated with DNase I. Ten micrograms of RNA was separated on a 1.5% agarose gel containing 2.2 M formaldehyde gel in MOPS (morpholinepropanesulfonic acid) buffer, as described previously (36). RNA was transferred to a Nytran membrane by vacuum transfer. Northern blot analysis was performed using $^{32}$P-labeled DNA probes for the E2F-responsive genes Cdk2, MCM3, RR1, and TS and the cellular actin gene.

## RESULTS

**Construction and isolation of recombinant viruses.** Although the HCMV IE86 protein has no structural homologs within the alpha- or gammaherpesviruses, members of the CMV family contain homologs that contain a high degree of sequence similarity at the C terminus of the viral protein. A multiple sequence alignment of the CMV IE86 protein homologs reveals several stretches of identical amino acids (Fig. 1). However, there is less identity when comparing the primate and the nonprimate IE86 protein homologs. The HCMV IE86 protein residue Q548 (denoted by an arrow in Fig. 1) is conserved throughout the primate and the nonprimate homologs. When combined with the presence of the Q548 amino acid mutation in the LXSN-IE86 cell line discussed previously, this suggests that the conserved Q548 residue may be important in the function(s) of the HCMV IE86 protein.

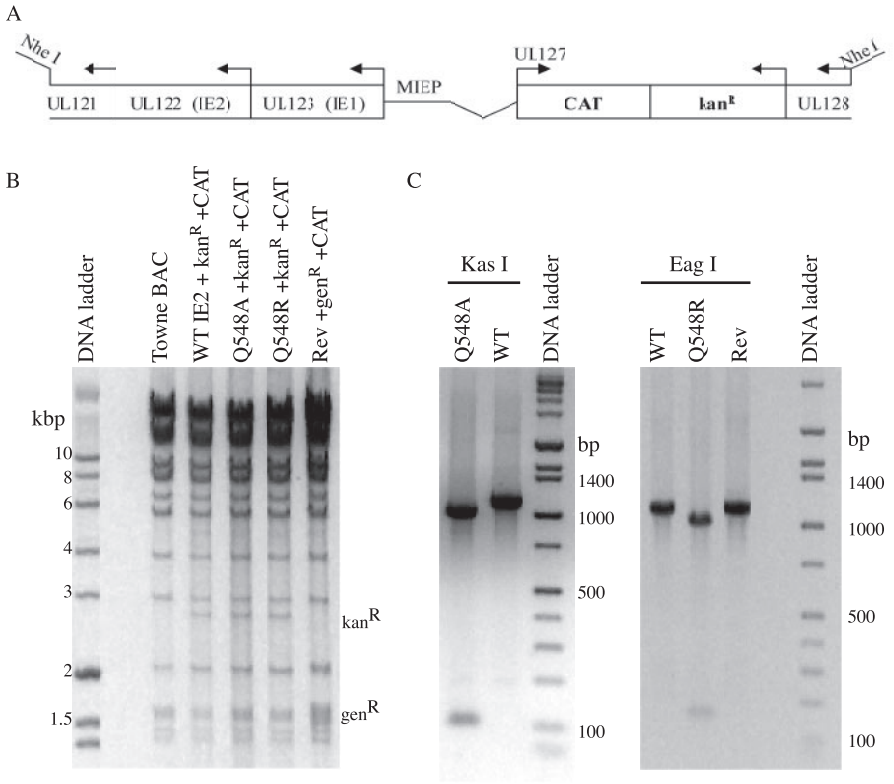


FIG. 2. Construction and confirmation of recombinant BACs. (A) Recombinant BACs were constructed by homologous recombination of a NheI-linearized DNA fragment with Towne BAC, kindly provided by F. Lui, University of California, Berkeley. UL121 and UL128 served as flanking regions to introduce targeted mutations into exon 5 of IE2. The UL127 locus was replaced by the CAT reporter, as described previously (27, 30). A kanamycin resistance (Kan$^r$) cassette was inserted between UL127 and UL128 for selection of recombinant BACs. (B) The integrity of recombinant BACs was verified by digesting BAC DNA with the HindIII restriction enzyme. (C) Exon 5 of IE2 was amplified from the recombinant BACs by PCR and digested with the indicated restriction enzyme. Successful recombination of the Q548A or Q548R mutation introduced a new KasI or EagI restriction site, respectively, compared to WT.

In order to test whether the Q548 residue is important for the function(s) of the HCMV IE86 protein during viral replication, recombinant BACs were constructed containing mutations of that amino acid. Single amino acid substitutions of Q548R, representing the mutation present in the LXSN-IE86 cell line, or Q548A, a neutral amino acid substitution for comparison, were introduced into a plasmid containing the entire MIE locus. In addition to the targeted mutation, a new restriction enzyme site was introduced into the sequence to facilitate screening of the mutants. The WT or mutant MIE genes were then transferred to a larger shuttle vector, containing HCMV sequences from UL121 to UL128. This shuttle vector was used to isolate a linear DNA fragment for recombination into Towne BAC (Fig. 2A).

The linear DNA fragment consists of the region between UL121 and UL128. The 5′ and 3′ ends of the fragment are identical to the sequence of Towne BAC and provide flanking regions for recombination. The UL127 locus has been replaced by the CAT reporter, as described previously (27, 30), such that it is expressed under viral early gene kinetics. A kanamycin resistance gene was introduced between the CAT reporter and the UL128 flanking region to facilitate selection of recombinant BACs. Recombinant BACs were generated containing WT IE86, or the Q548R or Q548A IE86 protein mutations. Additionally, a Rev BAC was constructed by replacing the Q548R mutation with WT sequence and replacing the kanamycin resistance gene with the gentamicin resistance gene.

Recombinant BACs were screened based on four factors to confirm that the BACs contained the correct sequences. First, *Escherichia coli* containing the recombinant BACs was screened for growth on chloramphenicol (Towne BAC), chloramphenicol and kanamycin (WT, Q548R, and Q548A BACs), or chloramphenicol and gentamicin (revertant BAC). Second, BAC DNA was digested with the restriction enzyme HindIII and separated on an agarose gel to verify that no large deletions or rearrangements of the HCMV genome occurred during recombination (Fig. 2B). Third, a region of exon 5 of IE2 was amplified by PCR from the recombinant BACs to generate an 1,100-bp PCR product. This PCR product was digested with the restriction enzyme KasI for the Q548A mutant or EagI for the Q548R mutant and compared to the WT or revertant PCR products digested with the same restriction enzyme to confirm the presence of the introduced mutation (Fig. 2C). Finally, the entire MIE locus of recombinant BACs was sequenced to verify that only the intended mutations in the IE2 gene were present (data not shown).

**Replication of recombinant viruses.** Recombinant BACs were initially transfected into HFF cells to determine whether they were able to replicate. Cells transfected with Towne, Q548A, or revertant BACs began to show CPEs at about day

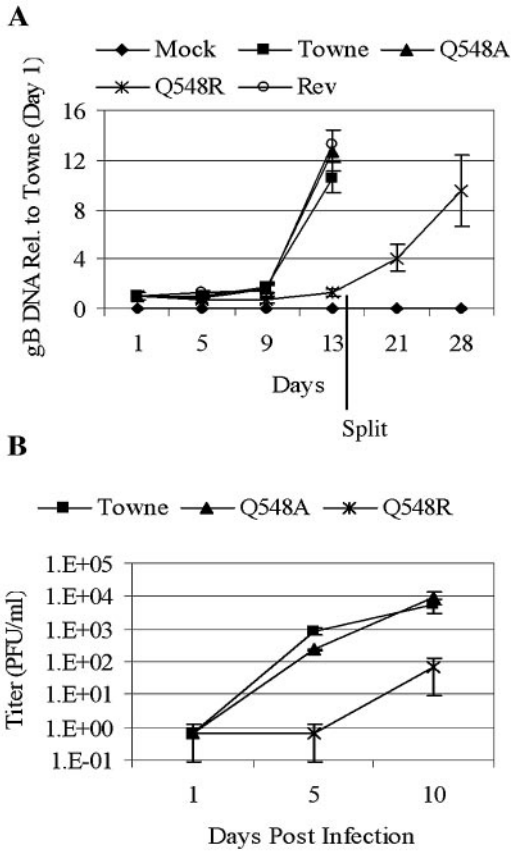


FIG. 3. Replication of recombinant viruses containing IE86 mutations. (A) HFF cells were transfected, in triplicate, with Towne, Q548A, Q548R, or Rev Q548R BAC DNA. Medium was changed on transfected cells every 4 days, and Q548R BAC-transfected cells were split 1:2 at day 14 to promote replication. Total DNA was harvested at 1, 5, 9, 13, 21, and 28 days posttransfection. Real-time PCR with primer/probe sets to detect HCMV gB DNA and cellular 18S rRNA genes was performed. BAC DNA replication was measured based on the amount of HCMV gB DNA, normalized to cellular 18S rRNA genes, relative to Towne-transfected cells at 1 day posttransfection. (B) HFF cells were infected, in triplicate, with equal DNA input of Towne, Q548A, or Q548R RV. Cells and supernatant were harvested at 1, 5, and 10 days postinfection, and triplicate samples were pooled and stored. A plaque assay was performed, in triplicate, with serial dilutions of stored virus, and plaques were counted at 10 to 14 days postinfection for Towne and Q548A RV-infected cells or 14 to 21 days postinfection for Q548R RV-infected cells.

5 and reached 100% CPE at about day 13. Alternatively, cells transfected with Q548R BAC did not show CPE until >21 days after transfection and never reached 100% CPE unless transfected cells were split and passaged for 6 to 8 weeks (data not shown). To quantify these results, total DNA was harvested from transfected cells and HCMV gB DNA was measured by real-time PCR. BAC DNA input is relative to Towne at day 1 after transfection, and all values represent the fold change in viral DNA from the input. Cells transfected with Towne, Q548A, or revertant BACs began to have an increase in gB DNA at day 9 and by day 13 had 10 to 14 times the amount of gB DNA that was present at day 1 after transfection (Fig. 3A). Cells transfected with Q548R BAC did not have a noticeable increase in gB DNA at day 13, so transfected cells were split 1:2 to promote DNA replication. By day 28, Q548R BAC-transfected cells had 9.5 times the amount of gB DNA that was present at day 1. The IE2 gene from the resulting Q548R RV was again sequenced to verify that only the intended mutation in the IE2 gene was present (data not shown).

Supernatants from transfected cells were collected and passaged, and recombinant viruses were frozen for future experiments. Equal DNA input for recombinant viruses was calculated by real-time PCR for HCMV gB DNA. A plaque assay was performed using the frozen recombinant virus stocks to determine their replication rate. HFF cells were infected with equal DNA input of Towne, Q548A, or Q548R RV equivalent to an MOI of approximately 0.0001. Infected cells and supernatants were collected at 1, 5, and 10 days postinfection (dpi), and the titers were determined by serial dilution on HFF cells. Towne RV reached a titer (PFU/ml) of $8.7 \times 10^2$ at 5 dpi and $5.3 \times 10^3$ at 10 dpi; Q548A RV reached a titer of $2.3 \times 10^2$ at 5 dpi and $9.3 \times 10^3$ at 10 dpi (Fig. 3B). Q548R RV exhibited smaller, nondistinct plaques that were not visible until about 21 days into the plaque assay (data not shown). A titer of $6.7 \times 10^1$ was reached at 10 dpi. The initial infection was also allowed to proceed to 14 and 21 dpi for the Q548R recombinant virus, but this had no significant effect on the final virus titer (data not shown). From these initial experiments, we conclude that the Q548R mutation in the IE86 protein results in a recombinant virus that replicates slower, and to a lower titer, than WT or a recombinant virus containing the Q548A mutation in the IE86 protein. Additionally, the phenotype of the Q548R RV is attributed to the mutation in the IE86 protein and not to possible mutations in other viral genes introduced during construction of recombinant BACs, since the revertant RV replicates like WT.

**Functional defects in the recombinant protein.** We next wanted to determine the functional defect in the recombinant virus containing the Q548R mutation in the IE86 protein. One or more functions of the IE86 protein could be impaired in this mutant, resulting in the impaired replication of the recombinant virus. These functions include the ability to negatively autoregulate transcription from the MIE promoter, transactivation of viral early genes, inhibition of cellular DNA synthesis, cell cycle arrest, and activation of cellular E2F-responsive genes. First, to determine whether the Q548R mutant IE86 protein was able to autoregulate expression of the MIE genes, we analyzed RNA and protein expression from HFF cells transfected with recombinant BAC DNAs. BACs were used for these experiments so that the results could be compared with those for a nonreplicating IE86 mutant, H446A/H452A (data not shown), which is predicted to be defective in autoregulation by the IE86 mutant protein (31, 32). Real-time PCR analysis was used to detect MIE RNA. The MIE RNA levels from HFF cells transfected with the Q548R BAC were within standard deviations of WT (Fig. 4A). MIE RNA from the H446A/H452A BAC was nearly threefold higher than that of WT. A Western blot of transfected HFF cells for the MIE proteins revealed that the Q548R IE86 mutant expressed 1.05 times the amount of the IE72 protein that WT did, while the H446A/H452A IE86 mutant expressed 4.44 times the amount of the IE72 protein that WT did (Fig. 4B). The IE86 protein was difficult to detect in HFF cells within a short exposure time, and at longer exposure times it was overshadowed by the

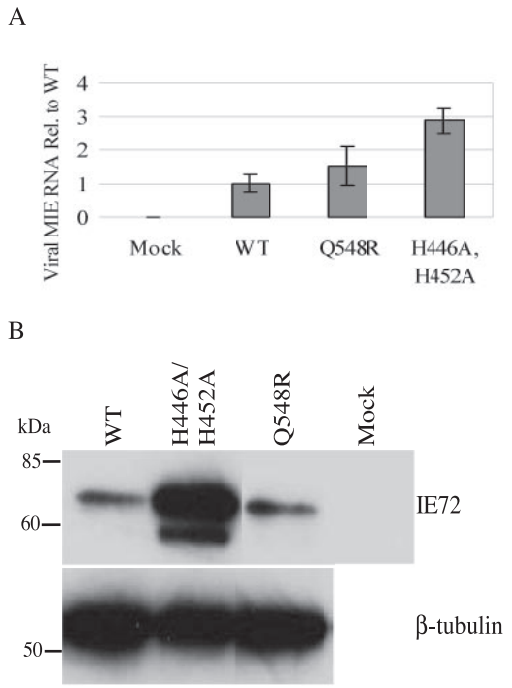


FIG. 4. Q548R mutant IE86 protein is able to negatively autoregulate expression from the MIE promoter. (A) HFF cells were transfected, in triplicate, with WT, H446A/H452A, or Q548R recombinant BAC DNA. Total RNA was harvested at 48 h posttransfection and converted to cDNA by reverse transcription. Real-time PCR was performed with primer/probe sets to detect HCMV MIE cDNA and cellular 18S complementary rRNA genes. MIE RNA transcription was measured based on the amount of HCMV MIE cDNA, normalized to cellular 18S complementary rRNA genes, relative to WT. (B) HFF cells were transfected with WT, H446A/H452A, or Q548R recombinant BAC DNA. Total protein was harvested at 24 h posttransfection. Western blot analysis was performed using antibodies to detect viral MIE proteins and cellular β-tubulin. In both figures, the H446A/H452A BAC contains mutations to the putative zinc finger of IE86, which is known to be defective in autoregulation of the MIE promoter (data not shown).

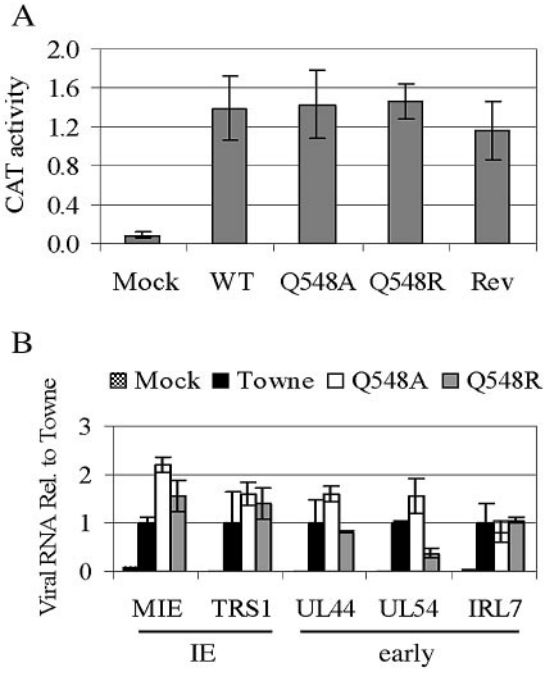


FIG. 5. Q548R mutant IE86 protein is able to transactivate viral early genes. (A) 293 cells were transfected, in triplicate, with β-Gal expression vector DNA and shuttle vector DNA containing WT, Q548A, Q548R, or Q548 Rev IE2 and the UL127 early viral promoter driving the CAT reporter. Total protein was harvested at 4 days posttransfection. A CAT assay was performed to determine the ability of the IE86 protein to transactivate the viral early promoter, and a β-Gal assay was performed to determine transfection efficiency. Results are reported as CAT activity (percent acetylation) per microgram of protein. (B) HFF cells were infected, in triplicate, with equal DNA input of Towne, Q548A, or Q548R RV at an MOI equivalent to approximately 0.001. Total DNA was harvested at 4 hpi; total RNA was harvested at 18 hpi and converted to cDNA by reverse transcription. Real-time PCR was performed with primer/probe sets to detect HCMV gB DNA; HCMV MIE and TRS1 IE cDNA; HCMV UL44, UL54, and IRL7 early cDNA; and cellular 18S complementary rRNA genes. HCMV IE and early RNA transcription was measured based on the amount of HCMV cDNA, normalized to cellular 18S complementary rRNA genes and gB DNA input, relative to Towne.

IE72 protein. However, the IE86 protein could be detected in 293 cells transfected with the Q548R BAC or HFF cells infected with the Q548R RV and was also expressed at levels similar to those of WT IE86 (data not shown). From these experiments, we conclude that the Q548R mutant IE86 protein is able to negatively autoregulate expression of the MIE genes similarly to WT.

The next function of the IE86 protein to be tested was the ability to transactivate viral early promoters. For this function, both endogenous viral genes and an artificial early promoter/reporter were utilized. The latter involved the CAT reporter driven by a modified UL127 promoter, which can be transactivated by the IE86 protein, as described previously (27, 30). To determine if the Q548R mutant IE86 protein is able to transactivate the UL127/CAT reporter, 293 cells were transfected with the shuttle vector shown in Fig. 2A, expressing the IE86 protein and containing the CAT reporter gene, as well as a β-Gal expression vector to determine transfection efficiency. CAT activities were similar from 293 cells transfected with WT, Q548R, Q548A, or revertant IE2/CAT shuttle vectors (Fig. 5A). Similar results were obtained when HFF cells were transfected with the IE2/CAT shuttle vector or with the recombinant BAC containing the CAT reporter (data not shown). To determine whether the Q548R RV was also able to transactivate endogenous viral early promoters, HFF cells were infected with either WT, Q548A, or Q548R RV. Viral RNA was analyzed by real-time PCR to detect viral IE and early genes, as well as viral DNA to normalize viral input. With two exceptions, the amount of viral RNA expressed from the Q548A and Q548R RVs did not differ from WT by more than twofold (Fig. 5B). The Q548A RV expressed 2.2-fold-more MIE RNA than Towne-infected cells, which explains the slightly higher levels of TRS1, UL44, and UL54 RNA that were also observed. The Q548R RV expressed 0.38-fold the amount of UL54 RNA that Towne-infected cells did. However, the Q548R RV expressed similar levels of MIE and TRS1 IE RNA, and UL44 and IRL7 early RNA (Fig. 5B). From these experiments, we conclude that the Q548R IE86 mutant is able to transactivate viral early genes, which is to be expected con-

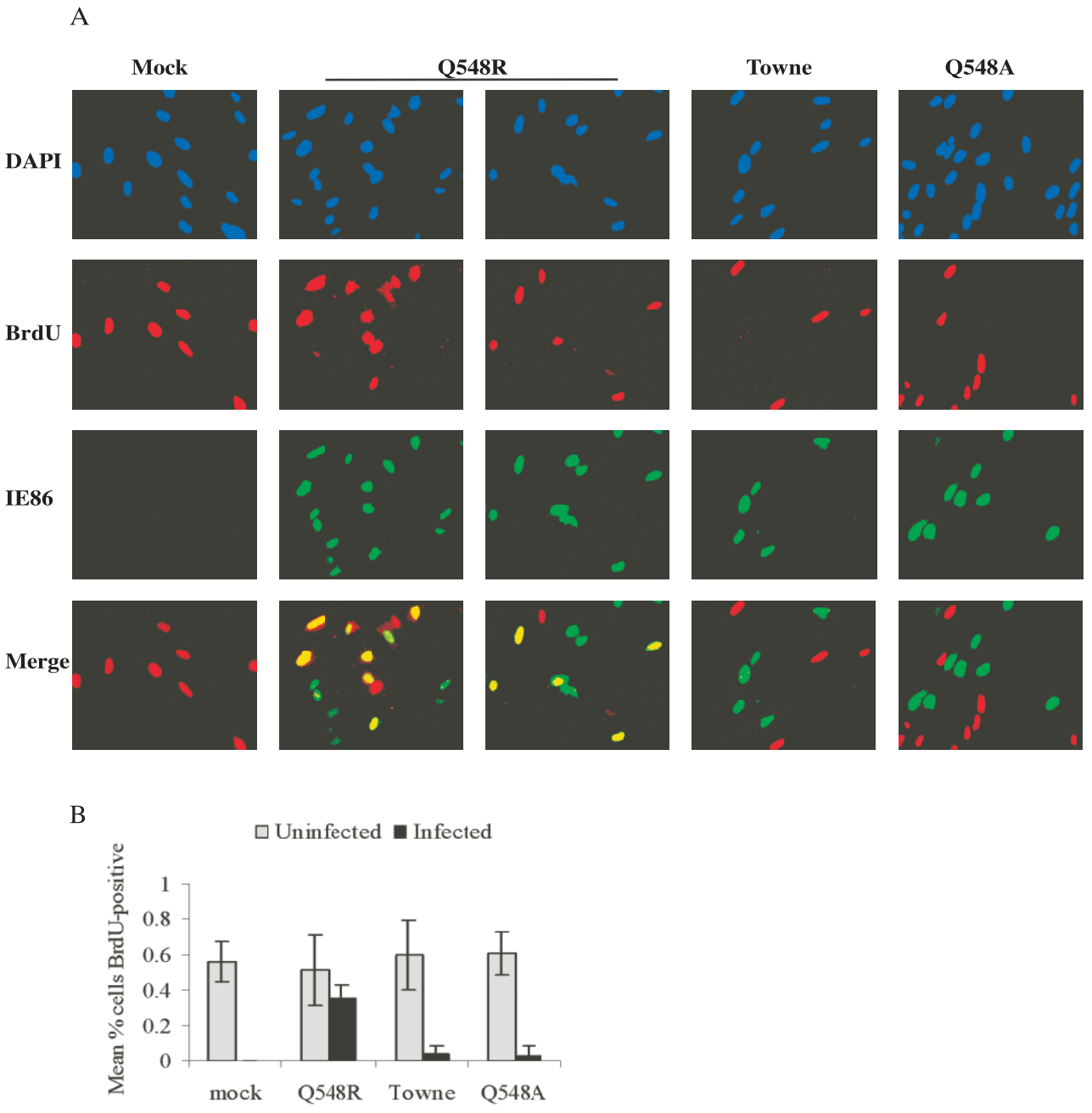


FIG. 6. Q548R mutant IE86 protein is unable to inhibit cellular DNA synthesis in infected HFF cells. (A) Serum-starved, contact-inhibited HFF cells were synchronized in $G_0$ for 48 h. Cells were infected for 6 h in low-serum medium with Towne, Q548A, or Q548R RV at an MOI of approximately 0.9. Cells were then split onto glass coverslips for release from contact inhibition and grown in high-serum medium, to promote cell cycle progression, plus BrdU, to detect DNA synthesis, for 42 h. Cells were fixed and permeabilized in methanol and acetone (1:1), stained with anti-IE86 and anti-BrdU antibodies, counterstained with DAPI, and imaged by fluorescent microscopy. (B) Ten fields, with at least 30 cells per field, from the above experiment were counted to determine the percentage of uninfected and infected BrdU-positive cells for each sample.

sidering that recombinant virus can be produced containing this mutation.

**Effect of the recombinant protein on cell cycle progression.** Since other functions of the Q548R mutant IE86 protein appeared relatively normal, we next tested the hypothesis that the Q548R mutant is unable to manipulate the cell cycle like WT. This includes the IE86 protein's ability to inhibit cellular DNA synthesis, to arrest the cell cycle in $G_1$/S, and to promote cell cycle progression from $G_0$ into $G_1$ by upregulating cellular E2F-responsive genes. To determine if the Q548R mutant IE86 protein was able to inhibit cellular DNA synthesis, a BrdU incorporation assay was performed on infected cells.

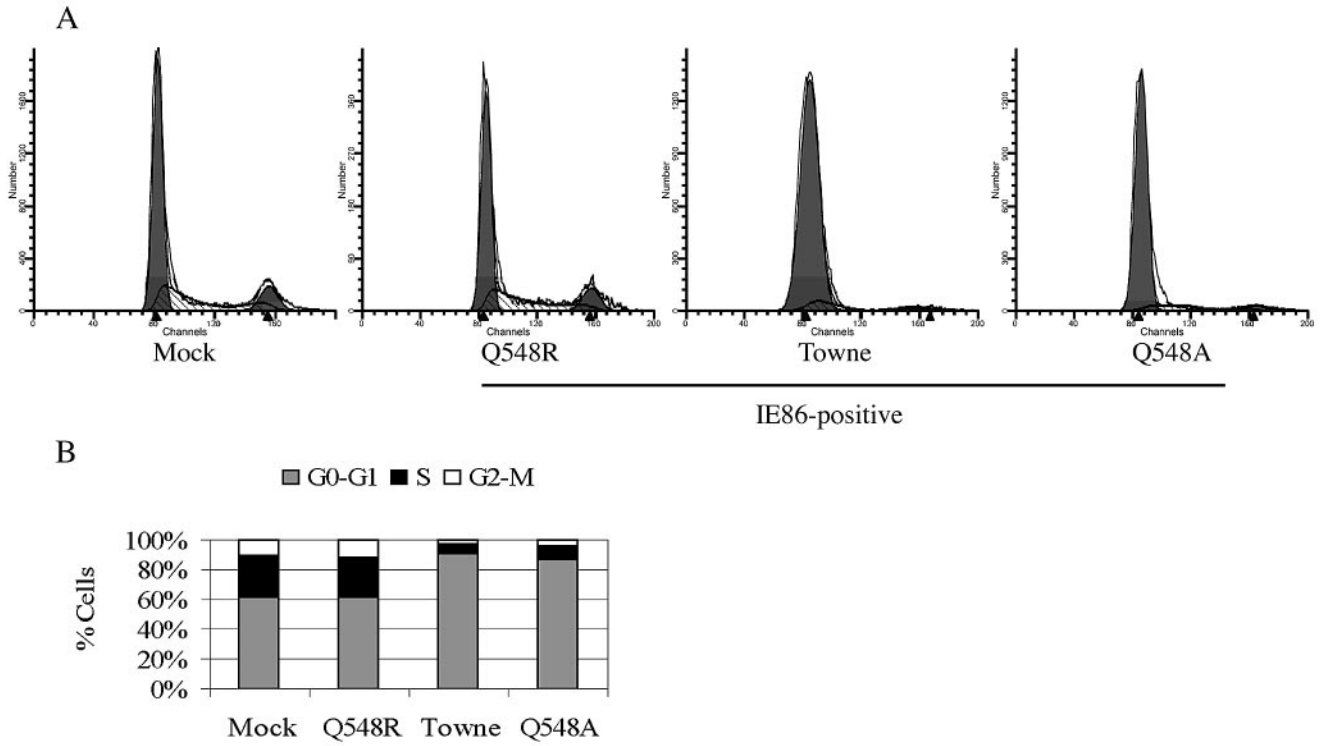


FIG. 7. Q548R mutant IE86 protein is unable to arrest cell cycle progression in infected HFF cells. (A) Serum-starved, nonconfluent HFF cells were synchronized in $G_0$ for 48 h. Cells were infected for 6 h in low-serum medium with Towne, Q548A, or Q548R RV at an MOI of approximately 0.9. Cells were then grown in high-serum medium to promote cell cycle progression for 42 h. Cells were permeabilized in 70% methanol, stained with anti-IE86 antibody, counterstained with DAPI, and subjected to FACS analysis. IE86-positive infected cells were sorted and counted based on DNA content. (B) Cell cycle profiles from the above experiment were analyzed using ModFit software to determine the percentage of infected cells in each phase of the cell cycle for each sample.

Confluent, contact-inhibited HFF cells were synchronized in the $G_0$ phase of the cell cycle to inhibit all cellular DNA synthesis. The synchronized cells were infected with Towne, Q548A, or Q548R RV at an MOI of 0.7 or mock infected. Infected cells were split onto glass coverslips in the presence of BrdU in order to promote and detect DNA synthesis. Infected cells were then fixed, permeabilized, and stained to detect infected cells (IE86), newly synthesized DNA (BrdU), and total nuclei (DAPI).

Approximately 40 to 50% of the cells were productively infected, based on IE86 protein staining, for imaging by fluorescent microscopy. In mock-infected cells, BrdU was visible in 56% of the cells (Fig. 6A and B). Similarly, in the Towne-infected sample, 60% of the IE86-negative (uninfected) cells were BrdU positive. As previously reported, IE86-positive Towne-infected cells exhibited little cellular DNA replication, due to the IE86 protein's ability to arrest the cell cycle and prevent cellular DNA replication (49). Viral DNA replication was visible at higher magnifications and appeared as punctate regions within the nucleus but was distinct from cellular DNA replication (data not shown). In the Q548A and Q548R RV-infected samples, similar percentages of IE86-negative cells were BrdU positive. However, while fewer than 5% of IE86-positive Towne or Q548A RV-infected cells exhibited cellular DNA replication, 36% of IE86-positive Q548R RV-infected cells were BrdU positive, indicated by yellow nuclei in the merged image (Fig. 6A and B). These data suggest that the Q548R mutant IE86 protein is unable to inhibit cellular DNA synthesis.

WT IE86 protein is able to arrest the cell cycle of p53-positive cells at the $G_1$/S transition, prior to cellular DNA replication (49). If a mutant IE86 protein was unable to arrest the cell cycle at this point, HCMV replication would be forced to compete with cellular DNA synthesis. To determine if the Q548R mutant IE86 protein is able to arrest cell cycle progression into S phase, FACS analysis was performed on infected cells to assess their cell cycle profile. Nonconfluent HFF cells were synchronized in $G_0/G_1$ and infected with Towne, Q548A, or Q548R RV at an MOI of 0.7 or mock infected. Infected cells were released from synchronization to promote cell cycle progression. Infected cells were then permeabilized and stained to detect infected cells (IE86) and DNA content (DAPI).

Cells that were infected at a 40 to 50% rate, based on IE86 protein staining, were sorted, and only IE86-positive (infected) cells were used for FACS analysis. In mock-infected cells, 38% of the cells progressed into S phase or $G_2$/M, based on DNA content (Fig. 7A and B). Alternatively, in Towne and Q548A RV-infected cells, which both inhibited cellular DNA synthesis, only 9% and 13% of the infected cells progressed into S phase or $G_2$/M, respectively. Consistent with the results from the previous experiment, 39% of the Q548R RV-infected cells progressed into S phase or $G_2$/M. These data suggest that the Q548R mutant IE86

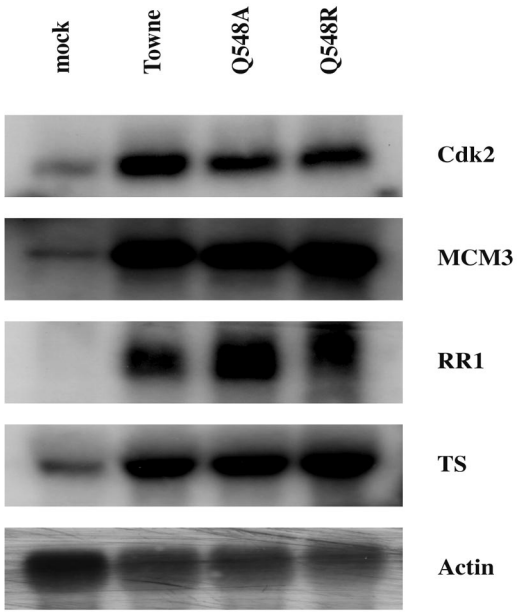


FIG. 8. Q548R mutant IE86 protein is able to upregulate E2F-responsive genes in infected HFF cells. Confluent, contact-inhibited HFF cells were infected, in triplicate, with equivalent CPE input of Towne, Q548A, or Q548R RV, equivalent to an MOI of approximately 0.3. Cytoplasmic RNA was harvested at 48 hpi and separated on an agarose-formaldehyde-MOPS gel. RNA was transferred to a Nytran membrane, and Northern blot analysis was performed using $^{32}$P-labeled DNA probes for the E2F-responsive genes Cdk2, MCM3, RR1, and TS and the cellular gene actin.

protein is unable to arrest the cell cycle at the $G_1$/S transition and subsequently inhibit cellular DNA synthesis.

**Upregulation of cellular E2F-responsive genes.** WT IE86 protein is able to promote cell cycle progression from quiescent cells in $G_0/G_1$ to the $G_1$/S transition by upregulating E2F-responsive genes (48). Since the cells in the previous experiment were nonconfluent, they could progress towards S phase on their own in the presence of serum. Therefore, it is possible that the Q548R mutant IE86 protein is unable to upregulate E2F-responsive genes in a quiescent cell. To determine if the Q548R mutant IE86 protein is able to upregulate E2F-responsive genes, Northern blot analysis was performed on infected cells. Confluent, contact-inhibited HFF cells were infected with Towne, Q548A, or Q548R RV at an MOI of 0.3 or mock infected for 48 h. Cytoplasmic RNA was harvested, and Northern blot analysis was performed using DNA probes for the E2F-responsive genes Cdk2, MCM3, RR1, and TS. In mock-infected cells, all tested transcripts were detected except RR1, which was at a lower steady-state level (Fig. 8). When the cells were infected with Towne, Q548A, or Q548R RV, all tested transcripts were present at higher levels than mock-infected cells, compared to the actin loading control. These data suggest that the Q548R mutant IE86 protein is able to upregulate E2F-responsive genes but is unable to inhibit cellular DNA synthesis.

## DISCUSSION

The HCMV BAC is arguably the most important tool made available for the analysis of essential viral genes. It is now possible to construct viral genomes containing lethal mutations in the essential IE86 protein. This was illustrated by studies that disrupted or introduced large deletions in the IE2 gene, resulting in a nonviable virus and confirming the accepted conclusion that the IE86 protein is critical for HCMV replication (34, 59). Nonviable viruses have also been constructed containing more-defined deletions of the IE2 gene, providing important insight into the function of transactivation, putative zinc finger, and helix-loop-helix domains (53). More importantly, crippled recombinant viruses can be isolated more quickly using HCMV BACs than with the traditional system of generating recombinant viruses. This makes it possible to determine which functions of the IE86 protein may be dispensable for viral replication. However, all cell cycle control studies to date that have resulted in crippled viruses employed the use of large deletions that affect multiple functions, thereby making it difficult to assign a particular function to a well-defined region of the IE86 protein (43, 54).

We have chosen to use a different approach to examine the functions of IE86 and the regions of the protein that are responsible for those functions. Rather than delete large regions of the protein, thereby potentially altering the conformation or stability of IE86, we introduced single amino acid substitutions into the IE2 gene based on sequence conservation and in vitro results. Using the BAC system, we have constructed recombinant HCMV containing the Q548R or Q548A mutation in the IE86 protein. Recombinant HCMV with the Q548A mutation replicates nearly identically to WT. In contrast, recombinant HCMV with the Q548R mutation replicates significantly slower, and to a lower titer, than WT.

Extensive study of the Q548R mutant IE86 protein has yielded important information about the function, and possibly the structure, of the IE86 protein. We have determined that the Q548R mutant IE86 protein maintains the ability to autoregulate transcription of MIE RNA and expression of the viral IE72 protein compared with a known autoregulation mutant. Similarly, the Q548R mutant IE86 protein can still transactivate viral early promoters, whether artificial or endogenous, a function that is critical for virus replication. The reduced level of the UL54 transcript, which encodes the viral DNA polymerase, does present potential problems, although this may simply be due to variation in the experiment, as all other early viral genes that were tested appear normal. It is possible that the difference in UL54 expression could result in the delay of viral DNA synthesis and viral replication seen in Fig. 3. However, UL54 expression is reduced only by slightly greater than two-fold from WT but still exists in the nucleus of the infected cells at significant levels compared to those for mock infection. It is also possible that the difference in UL54 expression could indicate a subset of early viral genes that are transactivated by IE86 through an alternate mechanism, which is defective in the Q548R mutant IE86 protein. While this may be true, there have been no published reports supporting this theory and it would require further investigation.

A recombinant virus containing the Q548R mutant IE86 protein lacks the ability to arrest the cell cycle at either the $G_1$/S transition, which would be expected in p53-positive cells, or at $G_2$/M, which occurs in cells lacking p53 (49). This virus cannot inhibit cellular DNA synthesis in nonconfluent HFF cells, where cell division is permissible, thus causing competi-

tion for DNA replication between the cell and the virus. Finally, a recombinant virus containing the Q548R mutant IE86 protein is able to upregulate cellular E2F-responsive genes in quiescent HFF cells, although it is unknown if the mutant protein is capable of activating all of the cellular genes that are necessary for progression towards S phase and replication of viral DNA.

Introducing a positively charged arginine residue, but not a neutral alanine residue, at the Q548 position near the C terminus of the IE86 protein abolished defined functions of the viral protein, while not affecting other functions. Furthermore, the fact that this partially functional protein is still detectable using multiple antibodies against the IE86 protein suggests that the defect is not due to a gross misfolding of the viral protein. Instead, we propose that the addition of a basic residue in the proximity of an acidic activation domain likely disrupts the interaction of the IE86 protein with a cellular or viral protein. This interaction may be responsible for the IE86 protein's ability to arrest the cell cycle in $G_1$, prior to S phase, and inhibit cellular DNA synthesis.

In addition to the IE86 protein, the HCMV tegument protein encoded by the UL69 gene has also been shown to arrest HFF cells in $G_1$ (17, 29). The mechanism by which UL69 arrests cell cycle progression is unknown, but it has been determined that UL69 is sufficient to arrest the cell cycle. Although our results indicating that a recombinant virus containing the Q548R mutant IE86 protein is unable to arrest the cell cycle or inhibit cellular DNA synthesis appear to contradict the UL69-mediated cell cycle arrest, this phenomenon can easily be explained. UL69 was sufficient to arrest cells in $G_1$ when it was artificially introduced into cells at high levels by transient transfection or transduction with a UL69-expressing retrovirus vector (29). Similarly, the IE86 protein was sufficient to arrest cells in $G_1$/S when it was artificially introduced into cells at high levels by transduction with an IE86 protein-expressing adenovirus vector (49). In the context of the HCMV virion, however, UL69 may be necessary for cell cycle arrest, but only with a functional WT IE86 protein (17). In our experiments, UL69 is present, but the IE86 protein contains the Q548R mutation and does not arrest the cell cycle at any point. Therefore, we propose that HCMV-mediated cell cycle arrest requires both UL69 and a functional IE86 protein in order to efficiently arrest HFF cells in $G_1$ and inhibit cellular DNA synthesis.

The point at which HCMV arrests the cell cycle has been the topic of much controversy over the past 10 years (4, 38, 46, 56). A recent study by Song and Stinski has established that there are two different arrest points (49). In a p53-null or p53-mutant cell, HCMV IE86 arrests the cell cycle after S phase, thus allowing cellular DNA synthesis but still preventing cell division. This arrest is therefore p53 independent, likely the result of anaphase-promoting complex (APC) complex or the proteasome failing to degrade cyclin B, which releases the cell cycle from $G_2$/M back into $G_1$ (44, 55). In a p53-positive cell, the HCMV IE86 protein arrests the cell cycle prior to S phase, thus inhibiting both cellular DNA synthesis and cell division. This arrest is therefore p53 dependent, although the mechanism for this arrest is unknown. Interestingly, if p53 is added to a p53-null or p53-mutant cell, HCMV IE86 now is able to arrest the cell cycle prior to S phase (49). This implies that both the p53-independent and p53-dependent cell cycle arrest pathways are capable of functioning in a p53-null or p53-mutant cell, and likewise, a p53-positive cell probably contains two functional arrest pathways, although the latter masks the former.

In HFF cells, which are p53 positive, the Q548R mutant IE86 protein is unable to arrest the cell cycle at any point. Therefore, we propose that this mutant protein fails to activate both the p53-dependent and p53-independent arrest pathways. This is important because it suggests either that the two pathways share a common starting point or that this single amino acid substitution disrupts multiple IE86 interactions. It is not known whether the Q548R IE86 protein is able to stabilize p53 and allow for its phosphorylation at serine 15, which occurs during the p53-dependent arrest, although the Q548R mutant IE86 protein expressed by the LXSN-IE86 cell line still interacts with p53 (2). Nor is it known whether the Q548R IE86 protein can affect the APC complex or proteasome, which likely occurs during the p53-independent arrest. However, it is known that, despite the mutant protein's failure to arrest the cell cycle, the Q548R RV is still able to replicate, albeit very slowly. This suggests that, while this particular function of the IE86 protein may be dispensable for viral replication, it is critical for efficient replication of the virus. The question remains whether the addition of drugs that artificially arrest the cell cycle, such as mimosine ($G_1$), aphidicolin ($G_1$/S), or hydroxyurea (S phase), could rescue the replication of this recombinant virus.

Our observation that the Q548R mutant IE86 protein is able to activate cellular E2F-responsive genes, thus likely promoting cell cycle progression from $G_0$ into $G_1$, but fails to arrest the cell cycle at any point presents an interesting paradox. Several viruses encode proteins that manipulate the cell cycle, including papillomavirus E6 and E7 and simian virus 40 large T antigen. The difference between these proteins and WT IE86 protein is that these other viral proteins possess the ability to transform cells, while only IE86 both promotes and then later inhibits cell cycle progression. Therefore, an IE86 protein that promotes, but fails to inhibit, cell cycle progression would also be expected to possess the ability to transform cells. The Q548R mutant IE86 protein seems to be inconsistent with that hypothesis. Although we did not perform traditional assays to determine if HFF cells transfected or infected with the Q548R mutant IE86 protein are indeed transformed, our observations do not support the hypothesis. The transfected/infected cells do not continue to divide and still respond to contact inhibition as expected. It seems possible then that, despite the Q548R mutant IE86 protein being able to transactivate viral early genes and cellular E2F-responsive genes, it may not be able to overcome contact inhibition or other cellular processes necessary to transform HFF cells. This would also explain why the Q548R RV replicates faster when infected cells are split, since the cells would naturally progress from $G_0$ towards S phase, and viral DNA replication could then occur, even though it would be in competition with cellular DNA synthesis.

In conclusion, we have constructed a recombinant HCMV containing a single amino acid substitution in exon 5 of the IE2 gene that results in a virus that replicates significantly slower than WT HCMV. The recombinant virus containing IE86 protein with the Q548R mutation retains several of the functions of the WT IE86 protein, including the ability to negatively autoregulate transcription from the MIE promoter, to trans-

activate viral early promoters, and to upregulate cellular E2F-responsive genes. Unlike the WT IE86 protein, the Q548R mutant IE86 protein lacks the ability to arrest cell cycle progression; thus, cellular DNA synthesis occurs in the infected cell. The failure of the Q548R mutant IE86 protein to inhibit cellular DNA synthesis is likely the cause of a lower rate of replication of the Q548R RV relative to Towne and Q548A RVs. The precise mechanism of this defect will be the subject of future studies.

## ACKNOWLEDGMENTS

We thank L.-F. Yee (University of Iowa) for the design and testing of primers and probe sets used in real-time PCR detection of viral early genes. We also thank A. Klingelhutz (University of Iowa), J. Meier (University of Iowa), and the members of the Stinski lab for critical review of the manuscript. Finally, we acknowledge the assistance of the University of Iowa Central Microscopy Research Facility and the University of Iowa Flow Cytometry Facility.

This work was supported by grant AI-13562 from the National Institutes of Health.

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