

Figure 3. Expression plasmids with the UL76-77 Luc sequence. (a) Diagram of expression plasmids with UL76-77 Luc sequence inserted downstream of the HCMV MIE promoter with or without a stop codon or a frame-shift in the UL76 ORF. (b) Western blot analysis of UL76 protein in 12.5% SDS-PAGE. HeLa cells were transfected with pCMVflagUL76-77Luc with or without insertion of a stop codon in the UL76 ORF and harvested at 48 h post transfection. To detect fusion protein with a flag epitope, antibody F3165 (Sigma) was used. Lanes: 1, pCMVflagUL76-77Luc; 2, pCMVflagUL76stop1-77Luc (stop1); 3, pCMVflagUL76stop3-77Luc (stop3); 4, pCMVflagUL76stop4-77Luc (stop4); 5, pCMVflag-frame-shift-UL76-77Luc (frame-shift). (c) Western blot analysis of UL76 protein in 5–20% SDS gradient gel electrophoresis. Lanes: 1, stop1; 2, stop3; 3, frame-shift. (d) Quantity of the UL77 gene transcripts with the expression plasmids. RNAs were analyzed with UL77 specific primers and probe by real-time RT-PCR. The assay was performed in triplicate, and the standard error of the mean was determined. RNAs were normalized to G6PD RNA, and each value was relative to the level of pCMVflagUL76-77Luc. (e) Western blot analysis of UL77 luciferase fusion protein. To detect fusion protein with a luciferase protein, antibody PM016 (MBL, Nagoya, Japan) was used. Lanes: 1, wt; 2, stop1; 3, stop3; 4, stop4; 5, frame-shift. (f) Effects of UL76 ORF translation on the luciferase activity. HeLa cells were transfected with the expression plasmids and pCMV-Renilla luc for standardization of the transfection efficiency and

the cells were harvested 48 h posttransfection. The relative luciferase activity (ratio of Firefly to *Renilla* luciferase activity) was calculated. Each transfection was performed in triplicate. Statistical analyses were done using STATA version 10 (Stata Corporation, <http://www.stata.com/>). doi:10.1371/journal.pone.0011901.g003

and harvested at the indicated times after infection. The viral RNAs were analyzed by real time RT-PCR. HCMV MIE (IE1/2), and UL44 gene primers and reporter probes were described previously [21]. The expression levels of the MIE gene for RUL76stop and RUL76 rev. were similar after infection by real-time RT-PCR and Western blot analyses (Fig. 5a and b). The UL44 (p52) gene expression with RUL76 rev. was a little higher than that with RUL76 stop at 2 d.p.i. by real-time RT-PCR and Western blot analyses, but not at 3 and 4 d.p.i. (Fig. 5a and b). The UL77 transcripts for RUL76stop and RUL76 rev. at 2 and 3 d.p.i. were similar in relative amount (Fig. 5a). However, the protein expression level of UL77 was approximately 3- and 2- fold higher for RUL76stop compared to RUL76 rev. at 3 and 4 d.p.i., respectively (Fig. 5b, lanes, 5 to 8).

To exclude the possibility that UL76 protein itself has an effect on the viral gene expression, HFF cells stably expressing either EGFP or the EGFP-UL76 fusion protein were selected and isolated as described in the Materials and Methods. Western blot analysis detected the EGFP-UL76 fusion protein (major band) (Fig. 5c). The slower migrating band (minor band) may represent a posttranslational modification of EGFP-UL76 fusion protein. After infection with RUL76stop, the expression levels of the viral proteins were compared at 1, 2, 3, and 4 d.p.i. As shown in Fig. 5d, constitutive expression of the UL76 protein did not down-regulate UL77 expression or expression of MIE, or early viral proteins.

From these results, we conclude that the UL76 ORF translation in the HCMV-infected cell significantly down-regulated the expression of the overlapping UL77 ORF in the HCMV- infected cell.

Effect of deletion of the UL76 ORF

To determine the effect of the UL76 gene on the viral growth, we constructed a recombinant virus with the UL76 gene deleted with a flag epitope fused to the N- terminus of the UL77 ORF (RdUL76) and the revertant virus (Rwt-R) as described in the Materials and Methods and shown in figure 4 (left panel). The

integrity of the recombinant BACs were checked by digestion with Hind III (data not shown) and the recombination was confirmed by sequencing of the PCR product (data not shown). Cells were infected with either RdUL76/flagUL77 (**dUL76**), or RflagUL77 (flagUL77) at a MOI of 3. The viral RNAs were analyzed by real time RT-PCR and the viral proteins were analyzed by Western blotting. The UL77 transcripts for RdUL76/flagUL77 and RflagUL77 were similar in relative amount at 2 d p.i. and only marginally different at 3 d p.i. (Fig. 6a). Deletion of the entire region upstream of UL77 caused an approximately 4 and 5- fold increase in the expression of the UL77 protein at 3, and 4 d.p.i., respectively (Fig. 6b, lanes, 5 to 8).

HFFs were also infected with wt-R, RdUL76, or RUL76 stop at high (3 PFU/cell) or low (0.01 PFU/cell) MOI. Virus titers from infected cultures were determined by the 50% tissue culture infectious dose (TCID₅₀) assay as described in Materials and Methods at 1, 4, and 5 or 1, 5, 7, and 9 d.p.i., respectively. At high MOI, RdUL76 and RUL76stop replicated similar to wt-R (Fig. 6c). At low MOI, RdUL76 and RUL76stop replicated slower than wt-R at 5 d. p.i., but by 7 and 9 d p.i. the infectious titers were similar (Fig. 6d). From these results, we conclude that UL76 is not essential for viral replication in HFF cells. An effect of a virion- delivered protein on the viral gene expression could be detected at a high MOI [12]. However, UL76 gene had an effect on the level of infectious virus production at 5 d p.i. at a low MOI, but not at high MOI. It has been reported that UL76 protein has a potential to suppress the MIE gene expression as a virion protein [12,20], but RT-PCR did not detect a negative effect and Western blot analyses detected a marginal effect only at 3 d p.i. (Fig. 6a and b).

Discussion

A majority of the ORFs in HCMV are nonessential for viral replication in cell culture. These nonessential ORFs likely encode

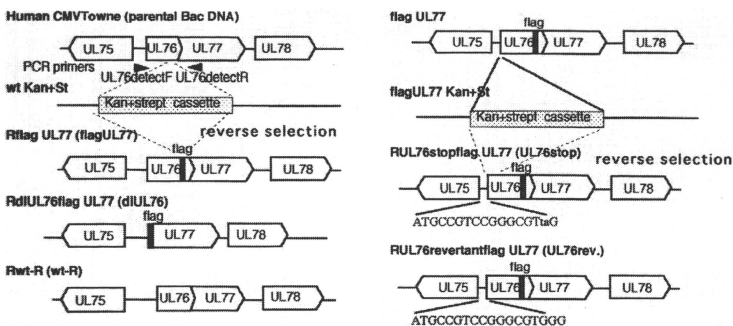


Figure 4. Structure of recombinant HCMV BAC DNAs. To construct the mutant BAC DNAs, a marker cassette containing the RpsI gene, conferring increased sensitivity to streptomycin, and the neomycin resistance marker to provide kanamycin resistance, was inserted into the UL76 ORF with or without a flag epitope fused to N-terminus of the UL77 ORF. Intermediate BAC clones were isolated based on resistance to kanamycin. In a second round of homologous recombination, the entire marker cassette was replaced with the mutated sequence by the counter selection using an oligo as described in the Materials and Methods. Lower case letters indicate mutated bases to insert the TAG stop codon in the UL76 ORF. doi:10.1371/journal.pone.0011901.g004

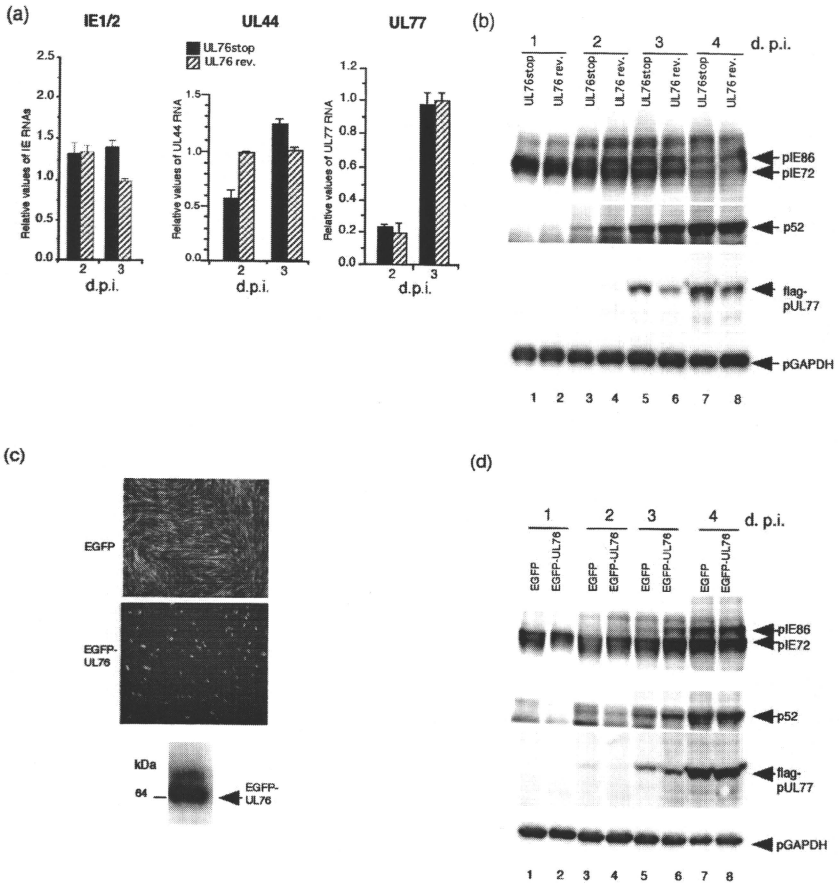


Figure 5. Analysis of the UL77 protein expression in cells infected with the recombinant virus with or without insertion of a stop codon downstream of the ATG start codon for UL76 ORF. (a) Quantity of viral major immediate early (IE1/2), early p52 (UL44), and UL77 gene transcription with the recombinant viruses. RNAs were analyzed with MIE, UL44, and UL77 specific primers and probes by real-time RT-PCR at 2, and 3 d. p.i. as described in the Materials and Methods. The assay was performed in triplicate, and the standard error of the mean was determined. HCMV RNAs were normalized to G6PD RNA, and each value was relative to the level of the RflagUL76revertantflagUL77 RNA at 3 d. p.i. (b) Western blot analysis of immediate-early pIE72 (UL123), pIE86 (UL122), early p52 (UL44), and flag-pUL77 proteins at the indicated times after infection with RUL76stopflagUL77; 2, 4, 6, and 8, RUL76revertantflagUL77 at an MOI of 1. Lanes: 1 and 2, 1 d.p.i.; 3 and 4, 2 d.p.i.; 5 and 6, 3 d.p.i.; 7 and 8, 4 d.p.i. (c) HFF cells stably expressing EGFP-UL76 fusion protein. The EGFP-UL76 fusion protein in HFF cells was detected by Western blot analysis using the polyclonal antibody against EGFP. (d) Western blot analysis for the detection of the immediate-early pIE72 (UL123), pIE86 (UL122), early p52 (UL44), and flag-pUL77 proteins in HFF cells stably expressing the EGFP (Lanes: 1, 3, 5, and 7) or EGFP-UL76 fusion protein (Lanes: 2, 4, 6, and 8) at the indicated times after infection with recombinant virus RUL76stopflagUL77 at an MOI of 1. Lanes: 1 and 2, 1 d.p.i.; 3 and 4, 2 d.p.i.; 5 and 6, 3 d.p.i.; 7 and 8, 4 d.p.i. doi:10.1371/journal.pone.0011901.g005

proteins with redundant functions or proteins that modulate viral replication. Global mutational analysis by constructing virus gene-deletion mutants classified UL76 as essential [1] or augmenting [4]

for viral replication. Our analysis indicates that UL76 is not essential. One of the roles of UL76 during viral infection might be to control the level of UL77 protein. Mutations in the UL76 ORF

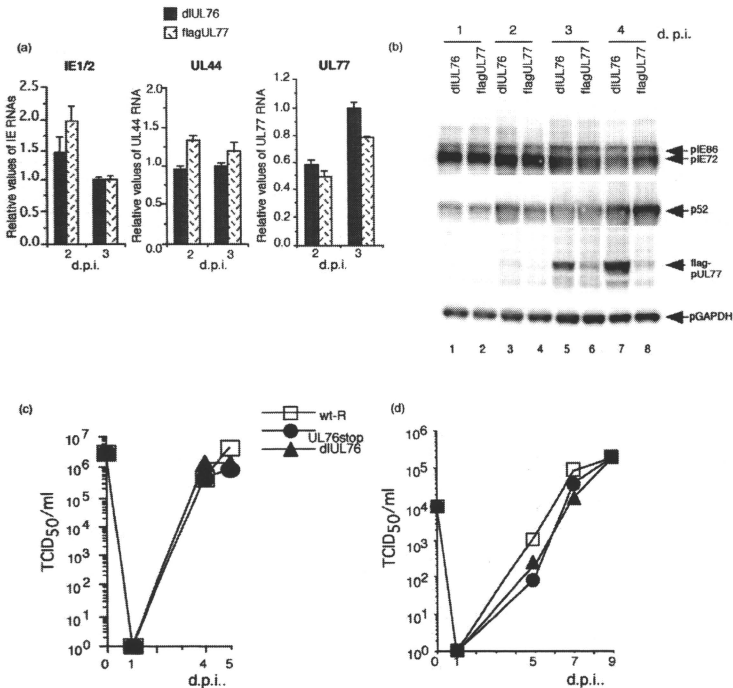


Figure 6. Effect of the UL76 on the viral growth in HFF cells. (a) Quantity of viral major immediate early (IE1/2), early p52 (UL44), and UL77 gene transcription with the recombinant viruses. RNAs were analyzed with MIE, UL44, and UL77 specific primers and probes by real-time RT-PCR at 2, and 3 d. p.i. as described in the Materials and Methods. The assay was performed in triplicate, and the standard error of the mean was determined. HCMV RNAs were normalized to G6PD RNA, and each value was relative to the level of the RdUL76flagUL77 RNA at 3 d. p.i. (b) Western blot analysis of immediate-early pIEB6 and pIE72 (UL122 and 123), early p52, and flag-pUL77 proteins at the indicated times after infection with RdUL76flagUL77(dUL76) or RflagUL77 (flagUL77) at an MOI of 1. Lanes: 1 and 2, 1 d.p.i.; 3 and 4, 2 d.p.i.; 5 and 6, 3 d. p.i.; 7 and 8, 4 d. p.i.; 1, 3, 5, and 7, RdUL76flagUL77; 2, 4, 6, and 8, RUL76flagUL77. (c-d) Growth curve of the w-R, RUL76stop, and RdUL76 at an MOI of 3 (c) or 0.01 (d). Virus titers were determined by the 50% tissue culture infectious dose (TCID₅₀) assay as described in the Materials and Methods. doi:10.1371/journal.pone.0011901.g006

by introducing stop codons and a frame-shift near the amino termini of the viral protein demonstrated that UL76 down-regulates the expression of UL77. Why UL77 is down-regulated during the viral replication cycle is not understood. Since UL76 protein is expressed in HCMV-infected cells [12,20], translation initiation complexes on the UL76-77 mRNA do not bypass the UL76 ORF. It is possible that UL77 interferes with virus replication if it accumulates too soon and to too high of a level before the viral DNA is ready for packaging. However, virus growth curves did not detect a decrease in virus titer at 5 p.i. after high MOI or at 7 and 9 d.p.i. after low MOI.

Multiple polycistronic mRNAs are frequently expressed in the HCMV-infected cell [13,14,15,16,17,18,19]. How the downstream ORF's are affected by the upstream ORF's is not well understood. The presence of an upstream ORF (uORF) inhibits initiation at downstream AUGs. The sole function of uORF may

be to down-regulate expression of the downstream ORF [22,23,24,25,26,27]. Our analysis indicates that UL76 plays a role in viral infection by significantly down-regulating the expression of the UL77 protein. This may be a mechanism that the virus uses to modulate the expression of viral gene products so that these viral proteins appear in the infected cell at the appropriate time and concentration for efficient viral replication.

How UL76 regulates the translation re-initiation of the UL77 ORF in the HCMV-infected cells is currently not understood. uORF usually consists of small peptides and efficient re-initiation occurs before the scanning ribosomes have dissociated from the mRNA [14,22,28]. The UL76 ORF makes a viral protein of approximately 38 kDa (325 amino acids). The large UL76 ORF suggest that the UL76-77 mRNA has an unusual translation re-initiation system. The region upstream of UL77 might contain a secondary structure, which facilitates UL77 ORF translation.

These data suggest that the UL77 protein needs to be expressed, but not to accumulate until there is sufficient viral DNA replication. Viral DNA accumulates slowly in the HCMV-infected cell between 24 and 72 h p.i. after a high MOI. The role of UL76 might be to modulate the level of UL77 gene product in the virus-infected cells.

Materials and Methods

Cells and virus

Primary human foreskin fibroblast (HFF) cells (KURABO INDUSTRIES LTD., Tokyo, Japan) were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum (Sigma, St. Louis, Mo.), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in 5% CO₂ as described previously [29]. To generate the HFF cells expressing the UL76 ORF, the retroviral system was used following the protocol from Nolan lab (http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html). The UL76 ORF was amplified by PCR from BAC DNA of HCMV Towne using a primer pair of XhoIUL76ORFF and HindIIIUL76ORFR. The sequences for the PCR primer are shown in Table S1. The PCR product was digested by restriction endonucleases Xho I and Hind III, cloned into the pLBC [30] (kindly provided by Dr. Kiem, Fred Hutchinson Cancer Research Center) with permission from Dr. Nolan, Stanford University) containing EGFP, at the corresponding restriction endonuclease sites, and DNA sequenced (Aichi Cancer Center Research Institute Central Facility). pLBC is a shuttle vector containing the EBNA ORF and the OriP sequence of EBV for constructing the recombinant retrovirus. A retrovirus stock was prepared by transfecting the shuttle vector, pLBC EGFP with or without UL76 ORF into the packaging cell line, Phoenix-GALV cells [31] (kindly provided by Dr. Kiem with permission from Dr. Nolan). HFFs were infected with retrovirus stock to generate a population of the cells expressing EGFP or EGFP-UL76 fusion protein under puromycin selection.

The virus titers of wild type (wt) HCMV Towne and the recombinant viruses were determined by standard plaque assays on HFF cells as described previously [8]. The titer of the recombinant viruses was also determined by GFP fluorescent foci in cells infected with serial dilutions. At various times after infection, cells and supernatant were collected and subjected to three freeze-thaw cycles. Virus titers were determined by the 50% tissue culture infectious dose (TCID₅₀) assay on HFF cells by detecting GFP foci in the 96 well. We used Reed-Muench method to calculate TCID₅₀. Wt and the recombinant viruses contain the GFP gene substituted for the dispensable, 10-kb US1-US12 region (US, unique short).

Enzymes

Restriction endonucleases were purchased from New England Biolabs Inc. (Beverly, MA.). High fidelity and expanded high fidelity Taq DNA polymerases were purchased from Invitrogen and Roche, respectively (Carlsbad, CA. and Mannheim, Germany) and RNasin and RNase-free DNase from Promega (Madison, WI.). The enzymes were used according to the manufacturers' instructions.

Southern blot analysis

Recombinant BAC DNAs were digested with restriction endonuclease BstBI, and then subjected to 0.6% agarose gel electrophoresis [5] and Southern blot analysis was performed as described previously [8]. The UL75 probe was described previously [21]. UL76 DNA was amplified from BAC-DNA of

HCMV Towne by PCR using the primer pair of UL76F (5'-CCGTCCGGGGCGTGGGGACGA-3') and UL76R (5'-CCG-TCCCAGATAGTCCAGGACAGA-3').

Northern blot analysis

Twenty micrograms of cytoplasmic RNA were subjected to electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and transferred to maximum strength Hybond N+ (Amersham). Northern blot analysis with IE1 probe was performed as described previously [8]. UL76 viral DNA was prepared as described above. UL77 and UL78 viral DNA were amplified from BAC-DNA of HCMV Towne as described above by PCR using the primer pair of UL77F (5'-CGTCTGG-CGCAGCAGCTGAGTTCGT-3') and UL77R (5'-CGTTCGAC-TCCAGAGAGAAAAGCAGTGC-3'), and UL78F (5'-CGTGA-GCCTGGTCAACCTGCTGACT-3') and UL78R (5'-ACGA-TGGAAAGAACCAGGCAAAGGCC-3'), respectively. A radioisotopic probe was generated by labeling with ³²P-dCTP.

Plasmids with UL76-77 sequence

Plasmids pCMVflagUL76-77HA, pCMVflag-frame-shift-UL76-77HA, and pCMVflagUL76-77-frame-shift-HA were constructed as follows. The UL76 to UL77 region with a flag epitope fused to the N-terminus of the UL76 ORF and a HA epitope fused to the C-terminus of the UL 77 ORF was amplified by PCR from BAC DNA of HCMV Towne with the primer pairs of EcoRIIflagUL76F and XbaIHAUL77R for pCMVflagUL76-77HA, EcoRIIframe-shiftflagUL76 and XbaIHAUL77R for pCMVflag-frame-shift-UL76-77HA, and EcoRIIflagUL76F and XbaIframe-shiftHAUL76R for pCMVflagUL76-77-frame-shift-HA. The primer sequences are shown in Table S1. The PCR products were digested by restriction endonucleases EcoR I and Xba I, cloned into the plasmid pCDNA3.1(+) (Invitrogen) at the corresponding restriction endonuclease sites, and DNA sequenced (Aichi Cancer Center Research Institute Central Facility).

Plasmids pCMVflagUL76-77Luc, or pCMVflag-frame-shift-UL76-77Luc was also constructed to estimate the effect of the UL76 sequence on the UL77 ORF translation. The luciferase gene was fused to the C-terminus of the UL 77 ORF at the NotI site located in the 480- nucleotide position from the ATG of the UL77 ORF. The luciferase gene was amplified by PCR from pSpLuc(+)-NF (Promega) with the primer pairs of NotIucF and XbaIucR (Table S1), digested by restriction endonucleases Not I and Xba I, cloned into the pCMVflagUL76-77HA or pCMVflag-frame-shift-UL76-77HA at the corresponding restriction endonuclease sites, and sequenced (Aichi Cancer Center Research Institute Central Facility).

To construct the UL76-77Luc plasmid with insertion of a stop codon into the UL76 ORF, site-directed mutagenesis was performed using a QuikChange XL site-directed mutagenesis system (Stratagene) with *PfuTurbo* DNA polymerase. The primers, each complementary to the opposite strands of the vector, were used to generate mutants. Sense-strand primer sequences are shown in Table S1. All of the mutations were verified by DNA sequencing.

Western blot analysis

To detect fusion protein with a flag or HA epitope, EGFP, luciferase, or cellular GAPDH as a loading control, antibody F3165 (Sigma), or 3F10 (Roche), A1122 (Molecular Probes, Eugene, OR.), PM016 (MBL, Nagoya, Japan), or MAB374 (Chemicon, Temecula, CA.) were used, respectively. To detect the pIE72 and pIE86 proteins encoded by IE1 and IE2, and pp52 encoded by UL44, we used primary mouse monoclonal antibodies

NEA-9221 (Perkin Elmer, Boston, MA.), and M0854 (Dako, Carpinteria, CA.), respectively. The procedure was described previously [7]. 5–20% SDS gradient gel was purchased from ATTO Corporation (Tokyo, Japan). Signal intensities were quantified with a LumiVision Image analyzer (Aisin/Taitec Inc., Tokyo, Japan).

Luciferase assays

All transfections were in triplicate on 24 wells using Lipofectamine and Plus reagent or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. HeLa cells were transfected with 1 µg of each expression plasmid with 50 ng of pCMV-*Renilla* luc, which serves as a control for the transfection efficiency and harvested 48 h post transfection. Cell lysates were then prepared and subjected to the Dual-Luciferase[®] Reporter Assay System according to the manufacturer's instructions (Promega). Data are averages of three independent transfection experiments. Statistical analyses were done using STATA version 10 (Stata Corporation, <http://www.stata.com/>).

Mutagenesis of HCMV BAC DNA

A rapid homologous recombination system in *E. coli* expressing bacteriophage lambda recombination proteins, *exo*, *beta*, and *gam* (provided by Dr. Court, NIH, MD), was employed as described previously [32]. BAC DNA of HCMV Towne was obtained from F. Liu (University of California, Berkeley, CA) [1]. To generate the recombinant HCMV BAC DNA of dUL76+Kan+FRT (see figure 1), the double-stranded DNA for recombination was amplified by PCR using the plasmid pACYC177 (NEB) as a template and the primer pairs of BACdUL76FRTfKanF and BACdUL76FRTfRkanR. The primer sequences are shown in Table S2. To generate the recombinant HCMV BAC DNA of wt Kan+St or flagUL77 Kan+St (see figure 4), the double-stranded DNA was amplified by PCR using the plasmid pRpsL-neo (Gene Bridges, Dresden, Germany) as a template and the primer pairs of BACUL76neoF and BACU76, or BACUL76stopneo+Stf and BACUL76stopneo+StR, respectively. The primer sequences are shown in Table S2. Plasmid pACYC177 or pRpsL-neo (Gene Bridges, Dresden, Germany) contains a kanamycin resistance (KanR) or KanR plus streptomycin sensitive gene, respectively. The amplified double-stranded DNAs for recombination contained a KanR gene flanked by the 34 bp minimal FRT sites (5'-GAAGTTCCTATTCTCTAGAAAAGTATAGGAACCTTC-3') [33], or RpsLneo gene (Gene Bridges, Dresden, Germany) and 70 bp of homologous viral DNA sequence. After digestion with Dpn I at 37°C for 1.5 h, the PCR product was gel-purified and transformed into the DY-380 containing the parental HCMV BAC DNA. After homologous recombination, the mutated BAC DNA containing the KanR plus FRT sequence, or RpsL-neo gene was resistant to kanamycin (see figures, 1 and 4).

To excise the KanR sequence from the mutated HCMV BAC DNA with FRT sequence, FRT mediated recombination was employed as described previously [7]. Plasmid pCP20 (provided by G. Hahn, Max von Pettenkofer Institute, Munich, Germany) was transformed into DH10B containing the recombinant HCMV BAC-DNA. HCMV BAC DNA without kanamycin was selected on LB plates containing ampicillin and chloramphenicol.

The reverse procedure was performed as described previously [34,35]. Since RpsL is a streptomycin sensitive gene, the mutated BAC DNA was selected on the basis of increased streptomycin resistance using a Counter Selection Modification kit (Gene Bridges). To construct BACflagUL77, BACdUL76-flagUL77, or BACwt-R, the oligo of BACUL76flagoligo-2, BACdUL76flagoligo-2, or BACwt-Roligo was used for the

reverse selection (see figure 4, left panel), respectively. The oligo sequences are shown in Table S2. To insert a stop codon with the recombinant virus with a flag epitope fused to N-terminus of the UL77 ORF, the RpsLneo gene was inserted into the UL76 of flagUL77 BAC DNA as described above (see figure 4, light panel). To generate RUL76stopflagUL77 or RUL76revertantflagUL77, the reverse selection was performed as described above using the oligo of BAColigoUL76stop or BAColigoUL76revertant, respectively. The oligo sequences are shown in Table S2.

PCR analysis

To select the recombinant BAC DNA, PCR analysis was performed using the following primer pair: UL76detectF: 5'-TAGCGGGTTACAAAAGTCGGCTCTCTGTCT-3' and UL76detectR: 5'-GCTCGGGGACGGCAGCAGCAGT-3'. The PCR cycling program was 1 cycle, denatured at 94°C, 2 min; 30 cycles, denatured at 94°C, 15 sec, annealed at 55°C, 30 sec, elongated at 72°C, 1 min, and 1 cycle, elongated at 72°C, 5 min. A PCR product was cloned into a pCR 2.1-TOPO TA cloning vector (Invitrogen) and sequenced to confirm the recombination and excision (Aichi Cancer Center Research Institute Central Facility).

Recombinant virus isolation

HFF cells were transfected with either 5 or 10 µg of each recombinant BAC in the presence of 2 g of plasmid pSVpp71 (HCMV tegument phosphoprotein pp71 driven by SV40 promoter) [36] by the calcium phosphate precipitation method of Graham and Van der Eb [37]. After 5 to 7 days of 100% CPE, the extracellular fluid-containing virus was stored at -80°C in 50% newborn calf serum until used.

Real-time RT-PCR analysis

For detection of RNA, whole-cell RNA was purified and then converted to cDNA with reverse transcriptase (RT) (Roche) as described previously [7]. The no reverse transcriptase control failed to detect any input viral or plasmid DNA and was similar to the mock control. Amplifications were achieved in a final volume of 25 µl containing PLATINUM Quantitative PCR SUPERMIX-UDG cocktail (Invitrogen). Each reaction mixture was described previously [38]. The forward and reverse primers and reporter probes for HCMV UL77 were designed using Primer Express (Applied Biosystems) as follows. UL77-566F: 5'-ACGATCCCTTTATCCGCTTTC-3'; UL77-633R: 5'-GGCATCTCGAACATGGTGT-3'; UL77-589 probe: 5'-FAM-ACCGATTTCGCGGGCGAGGTG-tetramethyl rhodamine (TAMRA)-3' (Nihon Gene Research Laboratories Inc., Sendai, Japan). HCMV IE1/2 and UL44 gene primers and reporter probes were described previously [5,34,38]. Thermal cycling conditions and a standard curve analysis were described previously [38]. Real-time PCR with G6PD primers and probes [39] was also performed to serve as an internal control for input RNA. Real-time RT-PCR assays were performed in triplicate. An arbitrary RNA in the isolated RNAs was set to 1.0 and a standard curve was constructed using serial dilutions of cDNA from the RNA set to 1.0. A constant amount of the RNAs was quantitated based on the standard curve.

Supporting Information

Table S1 PCR primers and oligos to construct the plasmids. Found at: doi:10.1371/journal.pone.0011901.s001 (0.03 MB DOC)

Table S2 PCR primer pairs and oligos to construct HCMV BAC DNAs.
 Found at: doi:10.1371/journal.pone.0011901.s002 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: HI MS TT. Performed the experiments: HI. Analyzed the data: HI MS. Contributed reagents/materials/analysis tools: HI YA. Wrote the paper: HI MS. Discussed experiments: MS TM SN SC TK TT. Supervised experiments: MS TT.

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