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Novel Method Based on "En Passant" Mutagenesis Coupled with a *Gaussia* Luciferase Reporter Assay for Studying the Combined Effects of Human Cytomegalovirus Mutations

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Human cytomegalovirus (HCMV) resistance to antivirals is a major problem in immunocompromised patients. Drug resistance is characterized by phenotypic testing or genotypic analysis of the phosphotransferase (UL97) and DNA polymerase (UL54) genes. However, genotypic assays require further characterization of unknown mutations in the drug resistance phenotype. Here, we describe a novel method for generating single or multiple mutations anywhere in the HCMV genome and for studying their effects on drug susceptibility. This method is based on cloning of the reference AD169 strain in a bacterial artificial chromosome and the use of "en passant" mutagenesis in bacteria to introduce mutations in recombinant HCMV without scar sequences. We also used this methodology to introduce the *Gaussia* luciferase reporter gene into the genome of the recombinant virus. To validate our system, the well-characterized single mutants with UL97 A594V and UL54 E756K mutations as well as the undescribed A594V/E756K double mutant were generated and their drug susceptibility profiles were determined by measuring the luciferase activity in cell culture supernatants. Drug susceptibility phenotypes for the A594V (8.2-fold increase in ganciclovir 50% effective concentration [EC_{50}]) and E756K (1.9-, 3.9-, and 3.0-fold increases in ganciclovir, foscarnet, and cidofovir EC_{50}s, respectively) mutants were similar to those previously reported, while the double mutant exhibited 10.8-, 4.1-, and 2.0-fold increases in ganciclovir, foscarnet, and cidofovir EC_{50}s, respectively. The combination of the *Gaussia* luciferase reporter-based assay with the markerless "en passant" mutagenesis methodology constitutes an efficient system for studying phenotypes with single or multiple HCMV mutations.

Human cytomegalovirus (HCMV) is a common pathogen that infects a large proportion of the population in the first years of life (1, 2). In immunocompetent individuals, infection is effectively controlled by the host immune system. In contrast, individuals with immature or compromised immunity may develop severe HCMV-associated diseases (3). In addition, HCMV is associated with indirect effects in solid-organ transplant recipients, leading to graft rejection (4).

All antiviral agents commonly used in the treatment of HCMV infections target the viral DNA polymerase (pUL54) (5). They include the nucleoside analogue ganciclovir (GCV), the nucleotide analogue cidofovir (CDV), and the pyrophosphate analogue foscarnet (FOS). GCV and CDV must be tri- and diphosphorylated, respectively, to be converted into their active forms. The viral kinase (pUL97) mediates the first phosphorylation of GCV, while cellular kinases perform the two phosphorylations of GCV monophosphate and CDV (6–8). In contrast, FOS does not require any phosphorylation to be active (9). Antiviral prophylaxis and preemptive therapy often are initiated in transplant recipients in attempts to decrease the occurrence of HCMV-associated diseases, which can be caused by primary infection, reactivation, or reinfection. Moreover, immunocompromised patients with severe HCMV-associated diseases often are treated with antivirals for long periods of time. One of the most problematic issues associated with prolonged antiviral treatment is the emergence of drug-resistant viral strains (10, 11), which can result in treatment failures. Mutations in UL97 and/or UL54 genes are involved in the selection of drug-resistant HCMV strains (5). Mutations in UL97 may cause resistance to GCV only, while mutations in UL54 may lead to resistance to one antiviral or even all three antivirals (10). The fact that UL54 contains polymorphisms (12, 13), at least outside its seven conserved domains, makes it even more important to determine which of the many mutations that can be detected in this gene confer drug resistance. Resistance to antivirals may be diagnosed either genotypically (i.e., by sequencing) or phenotypically. The genotypic method is rapid, but mutations with unknown significance remain problematic, and the degree of resistance conferred by combinations of mutations may be difficult to extrapolate. In contrast, phenotypic methods report levels of resistance but require growing the virus in cell culture, a process that can take many weeks. Moreover, the standard phenotypic method is still the plaque reduction assay (PRA), which is laborious and subject to variability (14).

For several years, the study and characterization of unknown mutations have been performed by recombinant phenotyping, which consists of introducing a mutated gene into a reference HCMV genome and assessing the drug susceptibility of the resulting mutant virus with a phenotypic assay, mainly the PRA. Several homologous recombination-based mutagenesis techniques with permissive eukaryotic cells have been developed (15, 16), but these methods are fastidious and time-consuming. The cloning of the whole HCMV genome as a bacterial artificial chromosome (BAC) has greatly improved the generation of mutant viruses by using

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the powerful recombination system of bacteria (17–19). However, most of these systems require cotransfection of additional plasmids for the expression of recombination enzymes. In addition, the marker cassette used for selection of the mutated recombinant virus often is excised through the concomitant use of flanking Flp recombination target (FRT) sites and a Flp recombinase, which leaves a residual recombination site next to the mutated gene (17). The presence of this scar sequence may interfere with gene expression and may prevent the generation of additional mutations using the same site-specific recombination target. An improvement was brought about with the *galK* selection-counterselection technique in a conditionally recombinogenic *Escherichia coli* strain, which allows introduction of mutations without leaving a scar sequence (20). However, this method has poor efficacy, especially in the counterselection step, and requires expensive growth culture medium (21). More recently, the Red recombination-based technique (22) has allowed for scarless generation of point mutations in more than one gene as well as the introduction of deletions and insertions of large DNA fragments potentially anywhere in the genome, in a timely manner.

Also, in an effort to increase the objectivity and rapidity of the phenotypic testing of recombinant viruses, different reporter genes, such as secreted alkaline phosphatase (15), firefly luciferase (23), and several types of fluorescent proteins (24–26), have been integrated into the HCMV BAC. The recently described *Gaussia* luciferase (GLuc) (27) is smaller (459 nucleotides) than the reporter genes used previously and thus is very attractive to avoid packaging limitations that might be associated with integration of an additional gene into the HCMV genome. Moreover, in contrast to the fluorescent proteins, this luciferase is naturally secreted and its activity can be measured in the supernatants of cells infected with the recombinant virus.

Herein, we describe the construction and characterization of a novel HCMV BAC system devoid of residual scar sequences into which we integrated the *Gaussia* luciferase reporter gene with a two-step Red-mediated recombination method (22). The well-characterized A594V and E756K mutations (10) were then incorporated, alone and in combination, into the HCMV UL97 and UL54 genes, respectively. The drug susceptibilities of the mutant viruses were then assessed using the *Gaussia* luciferase reporter-based assay.

MATERIALS AND METHODS

Cells and viral strains. Human foreskin fibroblasts (HFFs) and life-extended HFFs (LE-HFFs) (provided by T. Shenk, Princeton University, Princeton, NJ) were grown and maintained in minimal essential medium (MEM) (Gibco/Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS) (Gibco/Invitrogen, Burlington, Ontario, Canada). HFFs were used for transfection of the HCMV BAC and propagation of reconstituted viruses, as well as for drug susceptibility testing by the PRA. LE-HFFs, which constitutively express the catalytic subunit of human telomerase (28), were used to test the susceptibilities of the different recombinant viruses to antivirals in the *Gaussia* luciferase (GLuc) yield reduction assay. The wild-type (WT) AD169 strain of HCMV and its derived recombinant virus were used as reference strains in the PRA and the GLuc yield reduction assay, respectively. Recombinant HCMVs harboring the E756K mutation in the UL54 gene and/or the A594V mutation in the UL97 gene were generated by Red recombination, as described below.

BAC plasmid, plasmids, and bacterial strains. The original HCMV BAC plasmid (pHB5), derived from the AD169 reference strain, was provided by the laboratory of M. Messerle (Max von Pettenkofer Institut, Munich, Germany). In this plasmid, the BAC cassette replaces the US2-to-US6 gene region of HCMV, which is not essential for viral replication in cell culture (29). The original plasmid pHB5 has been transformed into *Escherichia coli* strain BW25141 (17). The pBKCMV82 plasmid contains the HCMV UL82 open reading frame (ORF), which codes for the viral protein pp71 and enhances the infectivity of viral DNA during infection (30). The pCMV-GLuc plasmid (New England BioLabs, Ipswich, MA) contains the *Gaussia* luciferase gene under the control of the cytomegalovirus (CMV) major immediate early (MIE) promoter. The pEP-Kan plasmid (kindly provided by N. Osterrieder, Cornell University, Ithaca, NY) contains a kanamycin resistance gene (*aphAI*) preceded by an I-SceI restriction site of 18 bp that is rarely found in bacterial genomes (31), which we referred to as the I-SceI-*aphAI* selection cassette. *E. coli* strain GS1783 (kindly provided by G. Smith, Northwestern University Medical School, Chicago, IL) was used in the two-step Red-mediated recombination procedure.

Electroporation of pHB5 into *E. coli* strain GS1783. The two-step Red-mediated recombination approach requires successive transient expression of the Red recombination proteins (i.e., Exo, Beta, and Gam λ phage proteins) and the I-SceI endonuclease. The GS1783 strain is the preferred host for this procedure since it contains both a heat-inducible promoter and an L-arabinose-inducible promoter, which control the expression of the Red recombination proteins and the I-SceI endonuclease, respectively (32). Therefore, pHB5 was first introduced into this bacterial strain. The *E. coli* GS1783 strain was made electrocompetent as previously described (33), and then 50 μl of the strain was mixed with pHB5 (150 ng; purified using a plasmid maxikit [Qiagen Sciences, Germantown, MD]), incubated for 30 min on ice, and transferred into a chilled 0.2-cm electroporation cuvette (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Bacteria were then resuspended in LB immediately after electroporation and were incubated for 1 h at 32°C, with agitation (225 rpm), for regeneration. Finally, bacteria were pelleted (4,000 × *g* for 5 min), plated onto LB agar containing 17 μg/ml of chloramphenicol (Sigma-Aldrich, St. Louis, MO), and cultured at 32°C for 24 to 48 h. An enzyme restriction analysis with EcoRI (New England BioLabs, Ipswich, MA) confirmed that all fragments of the electroporated pHB5 genome were similar to those of the original bacmid.

Integration of the *Gaussia* luciferase gene into pHB5. The *Gaussia* luciferase coding sequence driven by the CMV MIE promoter from pCMV-GLuc was integrated into pHB5 using a markerless two-step Red recombination protocol (32). To integrate the *Gaussia* luciferase reporter gene into pHB5, we chose regions of homologies that corresponded to the 50-bp regions at the end of both the US1 and guanine phosphoribosyltransferase (*gpt*) genes, as described in Fig. 1. The US1 gene is not essential for viral replication in cell culture (29, 34), and the *gpt* gene, which was used previously as a selection marker for introduction of the HCMV genome into the BAC by homologous recombination in HFFs (35), also was dispensable here. A unique restriction site (i.e., BamHI) was first chosen in the GLuc sequence to allow cloning of the I-SceI-*aphAI* selection cassette into this gene. The I-SceI-*aphAI* region of pEP-KanS was amplified using forward and reverse primers (see Table 1 for sequences) that annealed with the template plasmid (underlined in Table 1). In addition, the forward primer (pEP-Kan_GLuc_Fw) had a 5′ extension arm containing the BamHI restriction site (italic type in Table 1) and the 50 bp following this site in the GLuc gene sequence (duplicated sequence), whereas the reverse primer (pEP-Kan_GLuc_Rev) had a 5′ extension arm containing only the BamHI restriction site (italic in Table 1). The goal of duplicating the 50-bp region of the GLuc sequence is to excise the I-SceI-*aphAI* selection cassette during the second step of Red recombination. The PCR product generated and the pCMV-GLuc plasmid were both digested with BamHI (New England BioLabs, Ipswich, MA), gel purified (GenElute gel extraction kit; Sigma-Aldrich, St. Louis, MO), and ligated together using T4 DNA ligase (New England BioLabs, Ipswich, MA), to obtain the universal transfer construct (UTC). To allow integration of the GLuc gene

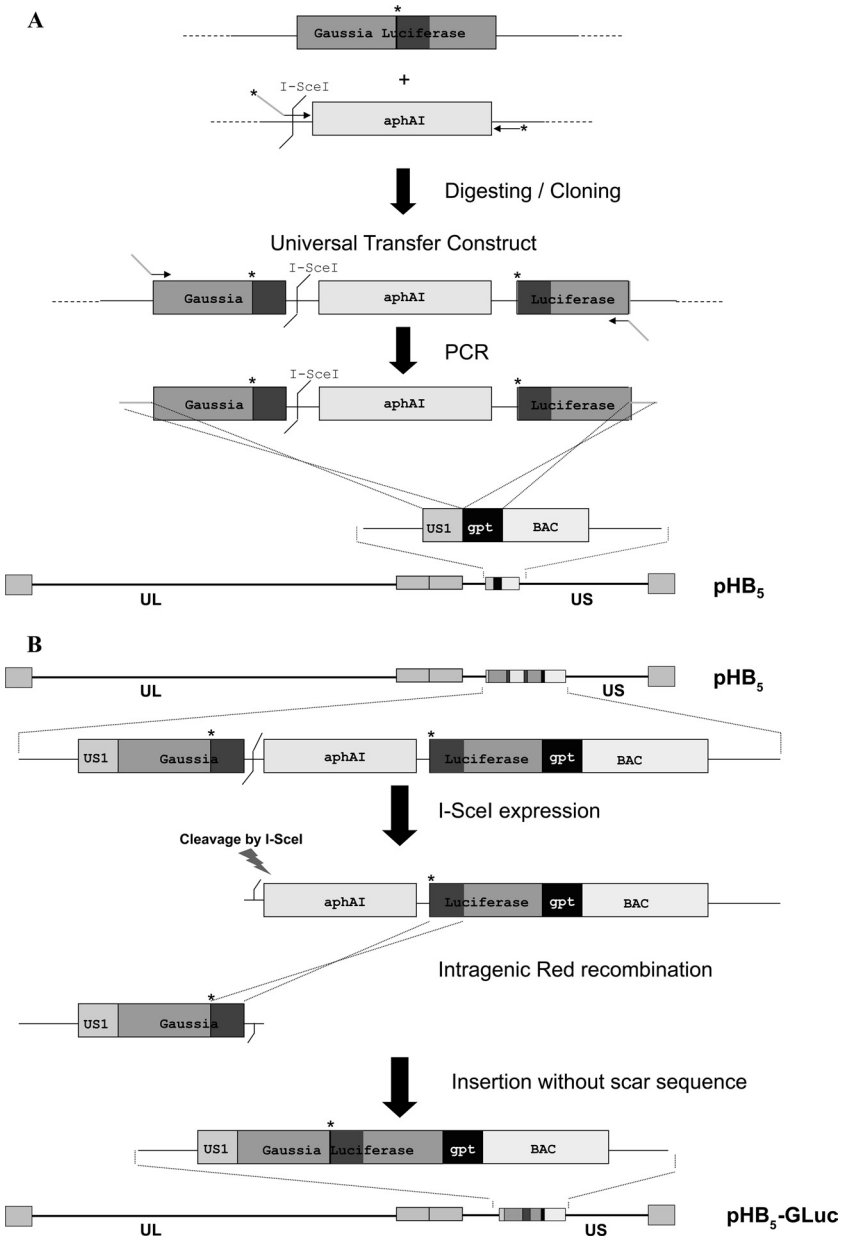

FIG 1 Schematic representation of the strategy used to introduce the *Gaussia* luciferase (GLuc) gene into pHB5. (A) A unique restriction site (i.e., BamHI) was chosen in the GLuc gene (*) followed by 50 bp, which was duplicated into the forward primer to amplify the kanamycin resistance cassette (*aphAI*) preceded by a I-SceI restriction site, to obtain the universal transfer construct (UTC). The UTC was then amplified by PCR with the last 50 bp of the US1 and *gpt* genes for the Red recombination procedure. (B) In the second step, *aphAI* was excised by expressing the endonuclease I-SceI followed by homologous recombination between the two duplicated regions of the GLuc gene. After integration of the GLuc gene, the US1 gene remained complete, whereas only the last 50 bp of the *gpt* gene remained in pHB5-GLuc. UL, unique long; US, unique short.

into pHB5 in the first step of Red recombination, its sequence was amplified from the UTC using primers (CUT_US1_Fw and CUT_gpt_Rev) (Table 1) with 5′ extension arms homologous to the 50-bp region of the recombination target sequence in either the US1 (forward primer) or *gpt* (reverse primer) gene. The PCR product generated (120 ng) was then electroporated into GS1783 bacteria made electrocompetent as described previously, with an additional step of incubation at 42°C for 15 min in a water bath shaker to induce expression of the Red recombination proteins (33). After electroporation, bacteria were regenerated, plated onto LB agar containing 50 μg/ml of kanamycin (Sigma-Aldrich, St. Louis, MO), and incubated at 32°C for 24 to 48 h. The I-SceI-*aphAI* selection cassette was then excised in the second step of Red recombination. An overnight culture of GS1783 bacteria was inoculated in 1 ml of LB containing chloramphenicol (17 μg/ml), at a ratio of 1:60, for 2 h at 32°C with agitation at 250 rpm. A 1-ml volume of LB containing both chloramphenicol (17 μg/ml) and L-arabinose (2% [wt/vol]) was then added to induce expression of the I-SceI endonuclease to cleave pHB5 at its specific recognition site, which is located before the kanamycin resistance gene. The culture was incubated for 60 min at 32°C with agitation at 250 rpm. Bacteria were then shifted to a water bath at 42°C for 30 min to induce the second step of Red recombination with the 50-bp duplicated region of the GLuc gene, which led to excision of the I-SceI-*aphAI* selection cassette as described previously

TABLE 1 Primers used for integration of the GLuc gene and different point mutations in pHB5 and pHB5-GLuc, respectively

Name	Template	Construct	Primer[a]
pEP-Kan_GLuc_Fw	pEP-Kan-S	I-SceI-*aphAI* plus GLuc duplicated sequence	5′-TTTT*GGATCC*AGCCACCATGGGAGTCAAAGTTCTGTTTGCCCTGAT CTGCATCGCTGTGGTAGGGATAACAGGGTAATCGATTT-3′
pEP-Kan_GLuc_Rev	pEP-Kan-S	I-SceI-*aphAI* plus GLuc duplicated sequence	5′-TTTT*GGATCC*GCCAGTGTTACAACCAATTAACC-3′
CUT_US1_Fw	pCMV-GLuc-*aphAI*	Universal transfer construct with homology arm	5′-GCCGCTAAGGCCGCATGCCCCTGCCGCCCAACTGAACACGCATAC CCCGCCGATGTACGGGCCAGATATACGC-3′
CUT_gpt_Rev	pCMV-GLuc-*aphAI*	Universal transfer construct with homology arm	5′-ATGAGCGAAAAATACATCGTCACCTGGGACATGTTGCAGATCCAT GCACGATTGCAGCACAGAAAAGCATCTTAC-3′
A594V_Fw	pEP-Kan-S	UL97 A594V point mutation	5′-AGGCGTTGCTCTTTAAGCACGCCGGCGCGGCCTGCCGCG**T**GTTGG AGAACGGTAAGCTCATAGGGATAACAGGGTAATCGATTT-3′
A594V_Rev	pEP-Kan-S	UL97 A594V point mutation	5′-ACAGGCGTCGGAGCAGTGCGTGAGCTTACCGTTCTCCAAC**A**CGCG GCAGGCCGCGCCGGCGCCAGTGTTACAACCAATTAACC-3′
E756K_Fw	pEP-Kan-S	UL54 E756K point mutation	5′-TACCCTGTGGACCCCGCCGACGTATACAGCGTCACGCTA**A**AGAAC GGCGTGACCCACCGCTAGGGATAACAGGGTAATCGATTT-3′
E756K_Rev	pEP-Kan-S	UL54 E756K point mutation	5′-CGCACCGAAGCACGCACAAAGCGGTGGGTCACGCCGTTCT**T**TAGC GTGACGCTGTATACGGCCAGTGTTACAACCAATTAACC-3′

[a] The underlined sequences correspond to the regions that anneal to the template. The BamHI restriction site used for cloning of the I-SceI-*aphAI* selection cassette in the GLuc sequence is shown in italics. The mutations introduced into the UL97 and/or UL54 genes appear in bold.

(32). Bacteria were finally transferred to 32°C for 2 to 3 h with agitation at 250 rpm, plated (dilution of 1:1,000) onto LB agar containing 17 μg/ml chloramphenicol, and cultured overnight at 32°C. After introduction of the GLuc gene, the US1 gene was still complete, while only the last 50 bp of the *gpt* gene remained in the HCMV BAC. The entire region between the US1 and *gpt* genes was finally sequenced to confirm correct insertion of the GLuc gene into pHB5, which we referred to as pHB5-GLuc. To verify the integrity of the pHB5-GLuc genome, 1 μg was digested with EcoRI overnight at 37°C and the profile was compared with the theoretical digestion profile, to ensure that no unwanted recombination events occurred. HFFs were transfected by electroporation with recombinant pHB5-GLuc and the pp71 expression plasmid (pBKCMV82) to enhance the infectivity of viral DNA, as described previously (30).

Generation of mutations in UL54 and/or UL97 genes in pHB5-GLuc. The E756K and A594V mutations were generated in the UL54 and UL97 genes, respectively, of pHB5-GLuc, alone or in combination, using the two-step Red recombination protocol (32) as described in Fig. 2. Primers used to generate the E756K mutation were E756K_Fw and E756K_Rev, and those used for the A594V mutation were A594V_Fw and A594V_Rev (Table 1). Both sets of forward and reverse primers annealed to the pEP-KanS template plasmid for amplification of the I-SceI-*aphAI* selection cassette (underlined in Table 1). In addition, each forward primer had a 5′ extension arm homologous to both the 40-bp segment downstream (segments a and b) and the 20-bp segment upstream (segment c) of the mutation (Fig. 2, step I). Each reverse primer had a 5′ extension arm homologous to both the 20-bp segment downstream (segment b) and the 40-bp segment upstream (segments c and d) of the mutation. The 40-bp segments at both terminal ends of the PCR product (segments a and b for the forward primer and c and d for the reverse primer) allowed its integration into pHB5-GLuc during the first step of Red recombination with target homologous sequences in the gene to be mutated (Fig. 2, step II). The 40-bp segments surrounding the mutation (segments b and c for both forward and reverse primers) corresponded to the duplicated sequence that was used in the second step of Red recombination for excision of the I-SceI-*aphAI* selection cassette after cleavage by the I-SceI endonuclease (Fig. 2, steps III to V). The procedures used for electroporation of the generated PCR product into electrocompetent *E. coli* strain GS1783 and successive induction of the expression of the Red recombination proteins and the I-SceI endonuclease were similar to those described above. Generation of the double mutant was performed by introducing successively the E756K mutation in the UL54 gene and then the A594V mutation in the UL97 gene. The introduction of mutations was confirmed by sequencing the entire UL54 and/or UL97 genes in mutated pHB5-GLuc and in DNA extracted from reconstituted mutant viruses.

Antiviral susceptibility testing. Testing of the susceptibility of recombinant viruses to antiviral agents was performed with HFFs or LE-HFFs seeded in 24-well plates, by the use of a PRA or a GLuc yield reduction assay (GLuc assay), respectively. Cells (90 to 100% confluence) were inoculated at 40 PFU (for the PRA) or a multiplicity of infection (MOI) of 0.001 (for the GLuc assay) and incubated for 90 min at 37°C in a 5% CO_2 atmosphere. Triplicate wells of infected cells were incubated with increasing concentrations of GCV, FOS, or CDV (all from Sigma-Aldrich, St. Louis, MO) in MEM plus 2% FBS containing 0.4% SeaPlaque agarose (Lonza, Rockland, ME) for 7 days (PRA) or in MEM plus 2% FBS for 6 days (GLuc assay). In the PRA, cells were fixed and stained and the PFU were counted under an inverted microscope, as described previously (14). In the GLuc assay, plates were agitated at 500 rpm for 30 s and 30 μl of cell culture supernatant was taken from each well in duplicate and transferred to a black 96-well plate (Microfluor 2 Black; Thermo, Milford, MA). A volume of 50 μl of coelenterazine substrate (NanoLight Technology, Pinetop, AZ), resuspended in acidified methanol at a concentration of 1 mM and then diluted 50-fold in PBS-5 mM NaCl as described previously (27), was added and the plate was agitated with an orbital shaker (orbit diameter, 0.1 mm) for 1 s. The luminescence was measured with a multilabel plate reader (Victor^3; PerkinElmer, Waltham, MA), with an acquisition period of 1 s. The concentrations of antivirals that reduced by 50% the number of plaques or the relative light unit (RLU) values, compared to control cells without drug (50% effective concentration [EC_{50}]), were determined for all reconstituted viral mutants and appropriate wild-type reference viruses. For the GLuc assay, the RLU values were plotted against the logarithm of the antiviral concentrations. A normalized dose-response inhibition curve was then fitted with a least-squares method, by the use of GraphPad Prism 5 (GraphPad Inc., La Jolla, CA), to calculate EC_{50} values. The normalization attributes the "top" (100%) and "bottom" values to the mean RLU values measured in the control wells without drug and to the lowest mean RLU values measured in the presence of the antiviral, respectively.

Replicative capacity testing. To determine the replicative capacity with the PRA, HFFs (90 to 100% confluence) seeded in a 24-well plate were inoculated in septuplicate at the specified MOI with the AD169 reference strain or pHB5-GLuc recombinant virus and were incubated for 90 min at 37°C in a 5% CO_2 atmosphere. The viral suspension was removed, and cells were incubated in fresh culture medium (MEM plus 2% FBS). The cell culture supernatant (30 μl) was collected in a separate well at daily

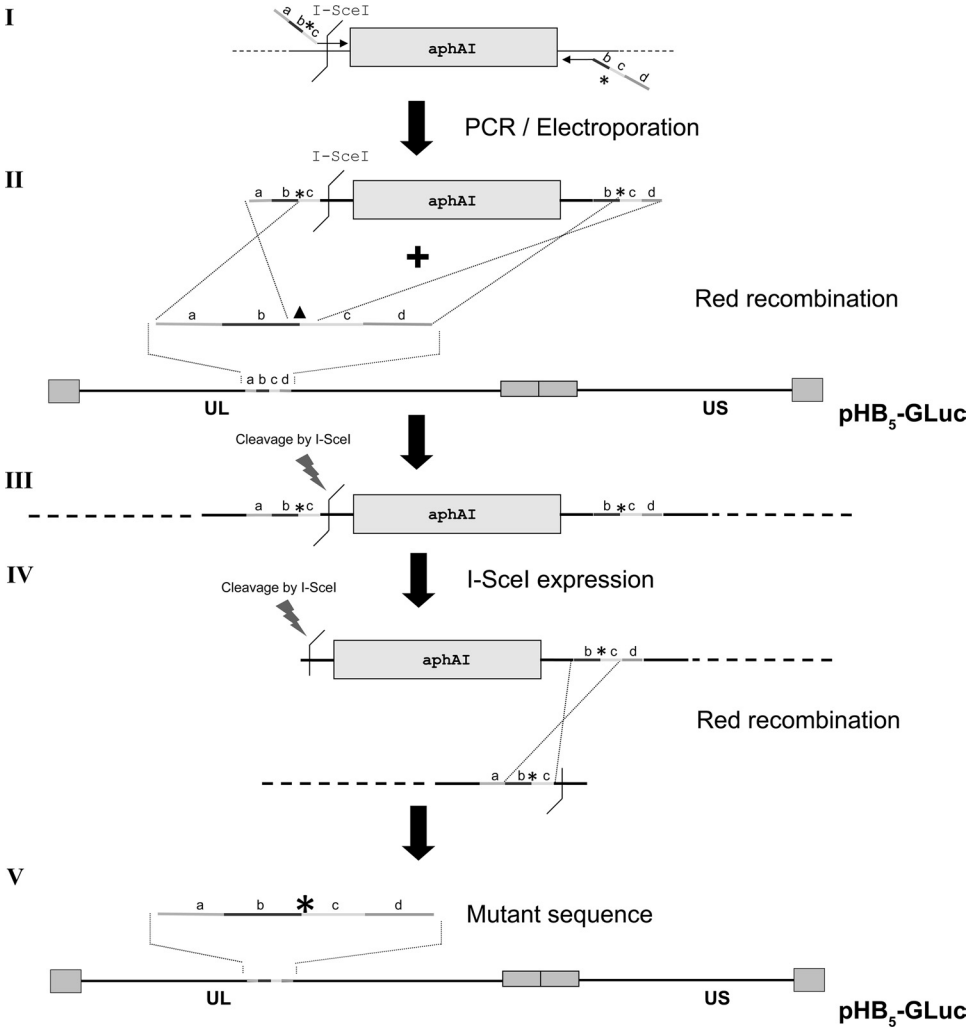

FIG 2 Schematic representation of the strategy used for the generation of point mutations in pHB5-GLuc. (I) Amplification of the kanamycin resistance cassette (*aphAI*) preceded by the I-SceI restriction site using primers containing 40-bp duplicated sequences (b and c segments) surrounding the mutated nucleotide (*) and 20-bp homology arms in each primer (a and d segments for forward and reverse primers, respectively). (II) Recombination with the target sequences in pHB5-GLuc (between the 40-bp sequences corresponding to the b-c and a-d segments, respectively) containing the wild-type nucleotide (▲), following heat induction of the recombination enzymes. (III) Induction of the I-SceI endonuclease with L-arabinose. (IV) I-SceI cleavage and intragenic recombination between duplicated sequences (b and c segments) following induction of the recombination enzymes. (V) Final mutant sequence. Each segment (labeled a to d) represents 20 bp. UL, unique long; US, unique short.

intervals for 6 or 7 days. The extracellular viral yield was determined by infecting new HFFs with serial 2-fold dilutions of each supernatant sample in duplicate. Infected cells were incubated for 7 days in MEM plus 2% FBS containing 0.4% SeaPlaque agarose, fixed, and stained, and the number of PFU in each well was counted.

RESULTS

Comparison of replicative capacity and susceptibility to antivirals by the PRA. The replicative capacity of pHB5-GLuc recombinant virus was compared with that of the AD169 reference strain by the PRA with HFFs (Fig. 3). By using both low (MOI, 0.1) (Fig. 3, left) and high (MOI, 1) (Fig. 3, right) MOIs, the infectivity growth curves were similar for the recombinant and reference viruses. Yields of both extracellular viruses decreased at days 1 to 2 and increased thereafter to reach a plateau at day 4 postinoculation. Susceptibilities of both viruses to GCV, FOS, and CDV were also evaluated by the PRA, to ensure that generation of the recombinant pHB5-GLuc did not alter the drug susceptibility profile. As shown in Table 2, EC_{50}s determined for the recombinant virus against the three drugs were similar to those for the AD169 reference strain. These values were also similar to those determined previously by using the same method (14).

Characterization of the *Gaussia* luciferase assay and susceptibility to antivirals in the yield reduction assay. To confirm that the *Gaussia* luciferase enzyme was effectively secreted and that its expression was directly related to the inoculum of recombinant virus used for cell infection, the replicative capacity of pHB5-GLuc was tested at increasing MOIs in LE-HFFs (Fig. 4). The enzymatic activity in the supernatant of infected cells was measured daily for 7 days. GLuc activity was readily detectable in the supernatant at 24 h postinoculation for a MOI as low as 0.001 (Fig. 4A), with RLU values 350-fold higher than those measured in the culture medium of uninfected cells (background). At day 7 post-

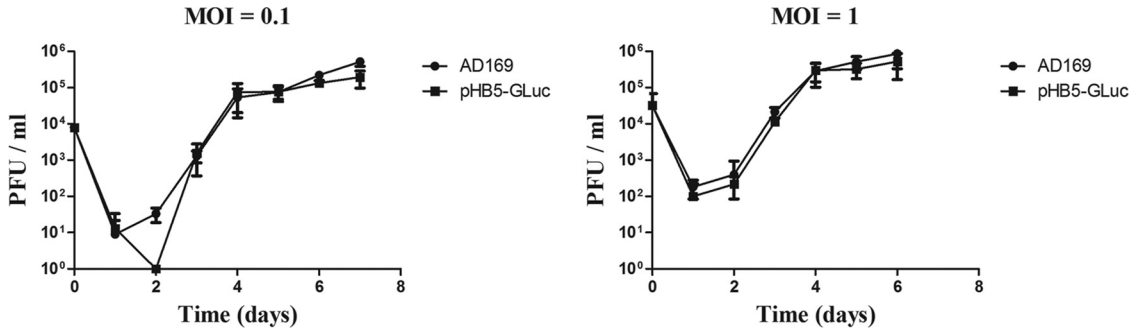

FIG 3 Replicative capacity of the HCMV AD169 reference strain and pHB5-GLuc recombinant virus at low (left) and high (right) MOIs, as determined with the PRA. HFFs were infected with both viral strains at MOIs of 0.1 and 1.0 for 90 min at 37°C in a 5% CO_2 atmosphere. The viral suspensions were removed, and cells were incubated with MEM plus 2% FBS. The cell culture supernatants were sampled daily for 6 or 7 days for the high and low MOIs, respectively. The extracellular viral yield was determined on new HFFs with the PRA. The day 0 values represent the infectivity of the input virus. Results represent the means ± standard deviation of three independent experiments.

inoculation, RLU values were 10- to 70-fold higher than those recorded on day 1. The enzymatic activity was also correlated with the viral inoculum used for cell infection and was linear up to a MOI of 0.5 ($r^2 > 0.9$) for 7 days (Fig. 4B). We selected MOIs between 0.001 and 0.005 as the optimal range to be used for cell infection, as RLU values were readily measured from the supernatants of cells infected with such inocula without any prior dilution.

We then evaluated the recombinant virus constructed with the *Gaussia* luciferase as a reporter gene in drug susceptibility testing. To determine the optimal day for the GLuc activity assay, we tested the growth kinetics of the pHB5-GLuc recombinant virus in the presence of different antivirals in cell culture supernatants collected 5 to 7 days postinoculation (Fig. 5A to C). Differences in RLU values started to be marked on day 5 for FOS and on day 6 postinoculation for GCV and CDV. In addition, the goodness of fit of the inhibition curves and the reproducibility of several experiments performed with the three antivirals indicated that day 6 postinoculation was the best time point for accurately determining EC_{50}s. The values obtained with the GLuc assay (Table 3) for the WT virus were slightly lower than those evaluated with the PRA (Table 2) but they followed the same trend, with CDV being the most active drug, followed by GCV and FOS. The EC_{50}s for all antivirals tested against the WT recombinant virus were also comparable to those previously reported for different reporter-based assay systems (15, 26).

Drug susceptibility of recombinant mutant viruses in the GLuc assay. To better validate the GLuc reporter-based assay system, we determined the susceptibilities of recombinant mutant viruses to GCV, FOS, and CDV. The A594V and E756K mutations have been shown to confer resistance to GCV only and to the three antivirals (GCV, FOS, and CDV), respectively (10). As expected, the recombinant virus harboring the A594V mutation showed an 8.2-fold increase in its EC_{50} for GCV, compared to the WT recombinant virus (Table 3). The EC_{50}s determined for the recombinant virus with the E756K mutation were 1.9-, 3.9-, and 3.0-fold higher than those of the WT virus for GCV, FOS, and CDV, respectively. The A594V/E756K double mutant showed 10.8-, 4.1-, and 2.0-fold increases in EC_{50}s for GCV, FOS, and CDV, respectively, compared to the WT virus.

TABLE 2 Susceptibility of the HCMV AD169 reference strain and pHB5-GLuc to antivirals in the PRA

Virus	Mean EC_{50} ± SD (μM)[a]		
	GCV	FOS	CDV
AD169	4.52 ± 0.62	63.10 ± 10.13	0.37 ± 0.07
pHB5-GLuc	4.59 ± 1.23	55.65 ± 7.99	0.39 ± 0.04

[a] $n = 3$ determinations.

DISCUSSION

Characterization of the role of unknown mutations in the resistance of HCMV to antivirals remains an important task to guide antiviral treatment. In this respect, the method of choice consists of introducing mutations found in clinical isolates into a reference HCMV genome cloned into a bacterial artificial chromosome (BAC) and testing the phenotype of the resulting recombinant virus. However, recombinant phenotyping methods described to date possess some weaknesses, such as the need to cotransfect additional plasmids for the expression of recombination enzymes (e.g., Flp recombinase [17]), the presence of a residual scar sequence (e.g., FRT site [18]) next to the mutated gene, and the need for expensive growth culture medium (21), and most methods are not time-effective. Moreover, the generation of several mutations in one or more viral genes is fastidious and time-consuming (15), thus limiting study of the combined effects of mutations. Here, we describe a novel and efficient method for the scarless generation of single or multiple mutations by Red recombination and testing for altered drug susceptibility of mutant recombinant viruses with a *Gaussia* luciferase reporter-based assay system.

Recombineering technology allows insertion or deletion of DNA sequences as well as sequential introduction of one or several point mutations potentially anywhere in the genome by using only 50-bp homologous regions, without leaving any scar sequences (22). We first used the Red recombination method to introduce the *Gaussia* luciferase gene into the HCMV BAC (pHB5). This reporter gene was chosen for its short sequence, to avoid packaging limitations for the large HCMV genome. The GLuc gene was integrated into the nonessential region from US1 to *gpt* of the pHB5 genome, which was previously used for introduction of the BAC (35). After introduction of the GLuc gene, the US1 gene remained unchanged but the *gpt* gene was almost completely removed, resulting in a genome size increase of less than 1 kb. The *Gaussia* luciferase is naturally secreted (36). After infection of cells with recombinant virus, enzyme activity can be mea-

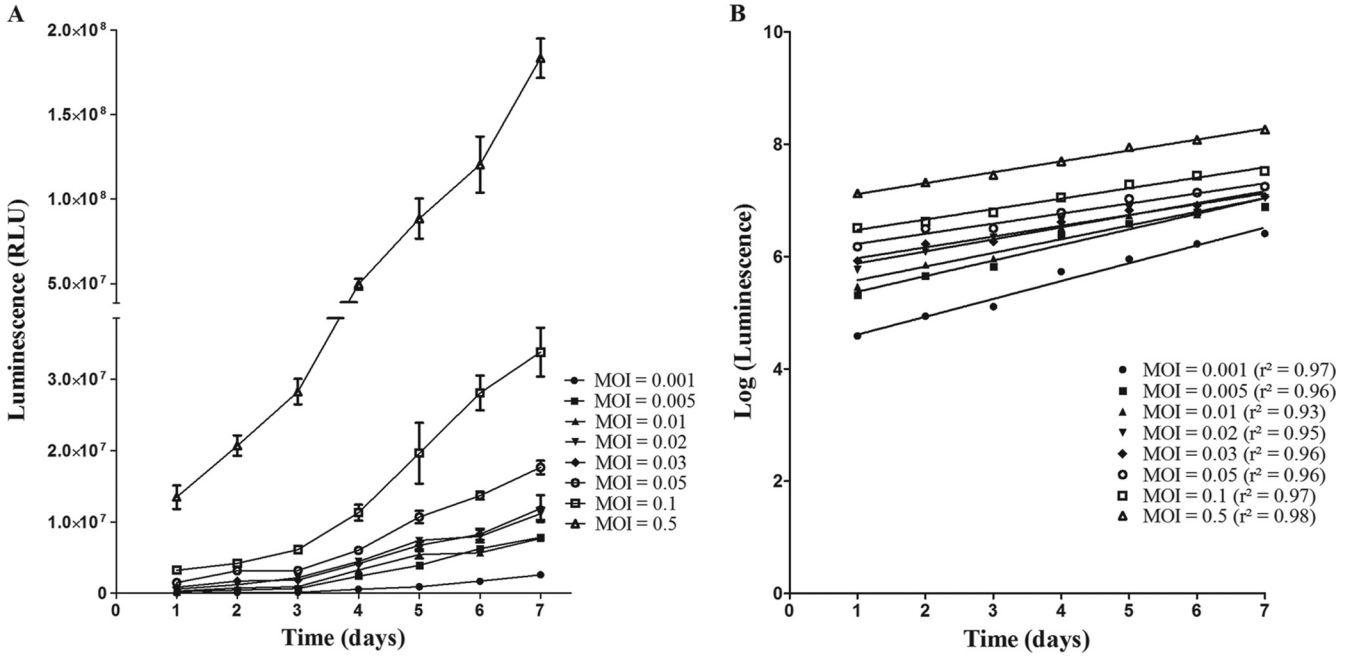

FIG 4 Replicative capacity of the pHB5-GLuc recombinant virus at increasing MOIs over time, as determined with the GLuc assay. LE-HFFs were infected with the recombinant virus at different MOIs ranging from 0.001 to 0.5. GLuc activity was measured in cell culture supernatants daily for 7 days. Supernatants of cells infected at the highest MOIs (0.5 to 0.01) were diluted 10-fold with PBS when RLU values exceeded 3×10^6 to 5×10^6 on the previous day. (A) The RLU values varied from 3×10^4 to 1.8×10^8 according to the MOIs and the incubation periods. (B) Data were linearized by logarithmic transformation. Regression coefficients remain acceptable up to a MOI of 0.5 ($r^2 > 0.9$) for an incubation period of 7 days. Results represent the mean ± standard deviation of sextuplicate determinations in one representative experiment.

sured directly in cell culture supernatants, which is more convenient than reporter-based assay systems using fluorescent proteins (24–26). GLuc demonstrated strong linear expression, which correlated with the infection time and the amount of input virus over a large range of MOIs. Compared with the secreted alkaline phosphatase reporter protein already used in HCMV recombinant phenotyping (15), GLuc has several advantages, such as greatly reduced assay times (1 to 10 min versus 1.5 to 2 h), increased sensitivity (20,000-fold higher sensitivity) (27), and a wider linear range (at 24 h postinfection, r^2 values are >0.9 for MOIs varying from 0.001 to 0.5, compared with r^2 values of 0.8 for MOIs varying from 0.005 to 0.04) (15). The major limitation of the GLuc reporter-based assay system is its flash kinetics, which require the use of a luminometer equipped with a built-in injector to perform the luminescence measurements (27).

Drug susceptibility testing with the GLuc yield reduction assay required optimal standardization to ensure low intra- and inter-assay variations. The latter were reduced through selection of a range of MOIs around 0.001 for cell infection, collection of at least 30 μl of supernatant for assays of enzyme activity, and selection of

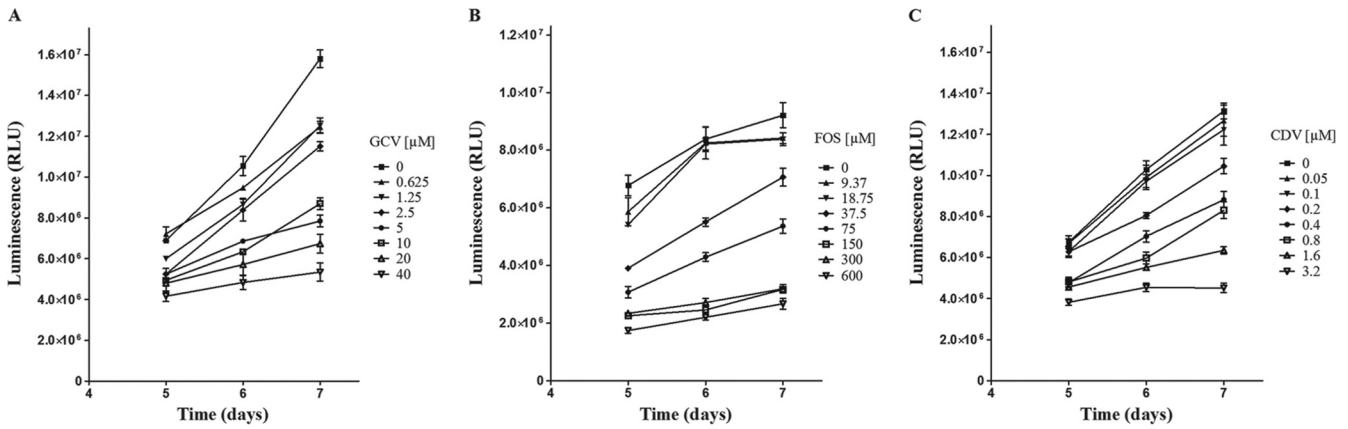

FIG 5 Representative growth kinetics of pHB5-GLuc in LE-HFFs exposed to ganciclovir (A), foscarnet (B), or cidofovir (C), in the GLuc yield reduction assay. LE-HFFs were infected with pHB5-GLuc at a MOI of 0.001 for 90 min at 37°C in a 5% CO_2 atmosphere. The viral suspension was removed, and cells were incubated with increasing concentrations of GCV, FOS, or CDV. At days 5, 6, and 7 postinoculation, cell culture supernatants were taken and GLuc activity was measured. Results represent the means ± standard error of sextuplicate determinations in one representative experiment.

TABLE 3 Susceptibility of WT and mutant pHB5-GLuc recombinant viruses to antivirals in the GLuc yield reduction assay

Mutation in pHB5-GLuc	Gene affected	Mean EC_{50} ± SD (μM) (fold increase from WT)[a]		
		GCV	FOS	CDV
WT	None	2.24 ± 0.36 (1.0)	51.14 ± 13.59 (1.0)	0.28 ± 0.09 (1.0)
E756K	UL54	4.19 ± 1.97 (1.9)	197.13 ± 50.72 (3.9)	0.85 ± 0.10 (3.0)
A594V	UL97	18.36 ± 6.76 (8.2)	ND	ND
A594V/E756K	UL97/UL54	24.18 ± 9.95 (10.8)	211.27 ± 91.26 (4.1)	0.55 ± 0.10 (2.0)

[a] $n = 3$ to 6 determinations. ND, not determined.

day 6 postinfection as the optimal day for measurement of GLuc activity. These optimizations resulted in more accurate determinations of EC_{50}s. The introduction of the GLuc gene into the genome of a HCMV reference strain by recombineering technology required a high degree of expertise in molecular biology, but the GLuc yield reduction assay has an objective readout and less variability than the standard PRA for drug susceptibility testing. However, the PRA requires less skill to perform, and it can also be used to test cell-associated clinical strains directly, rather than transferring a mutation to a reference strain.

We also generated two well-characterized mutations in the UL54 and UL97 genes, alone or in combination, to demonstrate the convenience of this system. The two-step Red-mediated recombination method has been already used for the generation of mutations in recombinant HCMV. However, the recombinant phenotyping was based on a fluorescent protein in fusion with an early-late HCMV protein, which did not allow the characterization of mutations elsewhere than in the viral DNA polymerase (26). Another system relied on measurements of secreted alkaline phosphatase activity under the control of the CMV MIE promoter (37), which, as mentioned previously, is less convenient than the *Gaussia* luciferase reporter-based assay system. Recombineering technology relies on the use of *E. coli* strain GS1783, which harbors the genes encoding the Red recombination proteins and the I-SceI endonuclease (32). This strain is a convenient host for HCMV recombinant phenotyping since it eliminates the need for additional plasmids for the expression of recombination enzymes, as is the case for several previously published methods (17, 18). The efficacy of Red recombination was superior to that of various previously published methods, particularly in the second selection step (counterselection), for which we observed at least 80% positive clones (data not shown), compared to the values of only 15 to 20% reported for other techniques (20, 21). Using the new methodology described here, we are usually able to generate a mutation in approximately 10 days. However, an additional 3 to 4 weeks is required before collection of recombinant virus following cell transfection, due to the slow growth of HCMV. The generation of the double mutant was performed in two successive Red recombination processes and took approximately 21 days (including sequencing analysis). Double mutants were previously generated by recombination between the whole genome of a reference HCMV strain digested at unique restriction sites and a transfer vector containing the mutated gene digested with the same restriction enzyme (15). However, the efficiency of the recombination events in eukaryotic cells was poor, and plaque purification of the mutated recombinant virus required several weeks. In addition, the generation of double mutants with this technique is limited by the need for unique restriction sites or their cloning in the genes of interest if they are not present. In contrast, the Red recombination system allows the introduction of several mutations potentially anywhere in the HCMV genome. We analyzed the combined effects on drug susceptibilities of two mutations in two different genes, in comparison with the effects of each mutation alone. The EC_{50} of GCV for the A594V/E756K double mutant corresponded roughly to the sum of the EC_{50}s determined for each single mutant, suggesting additive effects of these two mutations. The EC_{50}s of FOS and CDV for the double mutant remained similar to those determined for the E756K mutant.

Genotypic testing of HCMV-positive blood samples for drug resistance is becoming a standard approach in clinical practice (38). However, it requires prior characterization of the role of each mutation in drug resistance to generate a database (39) that would be available to physicians for more informed choices of treatment. In addition, the study of specific combinations of mutations found in clinical isolates could bring additional information with regard to their interactions and their influence on each other (e.g., additive or compensatory) in the context of drug resistance. The Red recombination system allows rapid easy generation of single or multiple mutations in one or more viral genes for the study of their combined effects on drug resistance. Drug susceptibility testing based on the *Gaussia* luciferase yield reduction assay has an objective readout and less variability than the PRA. It is clear that recombineering technology is likely to be adopted only by research reference laboratories with high levels of skill in molecular biology. However, identification of the role of unknown mutations in UL97 and UL54 genes in drug resistance by recombinant phenotyping is still needed. In addition, this technology will be particularly advantageous for characterization of mutations induced by novel antiviral agents acting on viral targets other than pUL97 and pUL54 (37, 40).

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