Targeted Mutagenesis of Guinea Pig Cytomegalovirus Using CRISPR/Cas9-Mediated Gene Editing

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ABSTRACT

The cytomegaloviruses (CMVs) are among the most genetically complex mammalian viruses, with viral genomes that often exceed 230 kbp. Manipulation of cytomegalovirus genomes is largely performed using infectious bacterial artificial chromosomes (BACs), which necessitates the maintenance of the viral genome in *Escherichia coli* and successful reconstitution of virus from permissive cells after transfection of the BAC. Here we describe an alternative strategy for the mutagenesis of guinea pig cytomegalovirus that utilizes clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated genome editing to introduce targeted mutations to the viral genome. Transient transfection and drug selection were used to restrict lytic replication of guinea pig cytomegalovirus to cells that express Cas9 and virus-specific guide RNA. The result was highly efficient editing of the viral genome that introduced targeted insertion or deletion mutations to nonessential viral genes. Cotransfection of multiple virus-specific guide RNAs or a homology repair template was used for targeted, markerless deletions of viral sequence or to introduce exogenous sequence by homology-driven repair. As CRISPR/Cas9 mutagenesis occurs directly in infected cells, this methodology avoids selective pressures that may occur during propagation of the viral genome in bacteria and may facilitate genetic manipulation of low-passage or clinical CMV isolates.

IMPORTANCE

The cytomegalovirus genome is complex, and viral adaptations to cell culture have complicated the study of infection *in vivo*. Recombineering of viral bacterial artificial chromosomes enabled the study of recombinant cytomegaloviruses. Here we report the development of an alternative approach using CRISPR/Cas9-based mutagenesis in guinea pig cytomegalovirus, a small-animal model of congenital cytomegalovirus disease. CRISPR/Cas9 mutagenesis can introduce the same types of mutations to the viral genome as bacterial artificial chromosome recombineering but does so directly in virus-infected cells. CRISPR/Cas9 mutagenesis is not dependent on a bacterial intermediate, and defined viral mutants can be recovered after a limited number of viral genome replications, minimizing the risk of spontaneous mutation.

Human cytomegalovirus (HCMV) is the most common congenital viral infection and the leading preventable cause of long-term neurologic disability (1). HCMV is readily transmitted across the placenta, and congenital infection occurs in 0.5 to 2% of births in the United States (2). The cytomegaloviruses (CMVs) are among the most genetically complex mammalian viruses and are widely believed to have coevolved with their hosts. The ~235-kbp HCMV genome encodes hundreds of proteins and numerous noncoding RNAs (3, 4). HCMV replication is restricted to cells of human origin, and many viral genes have adapted to support replication in the virus's natural host. Guinea pig CMV (GPCMV) is a small-animal model of congenital CMV infection; GPCMV is naturally transmitted across the placenta, and similarities between human and guinea pig gestation and placentation make this rodent a powerful model for the study of infection during pregnancy (5–7).

Manipulation of the large CMV genome is a technical challenge. Specific changes to the viral genome can be introduced by homologous recombination-driven insertional mutagenesis in mammalian cells (8, 9). While this methodology was highly successful in alphaherpesviruses, the larger genome and slower replication kinetics of CMV caused insertional mutagenesis to be inefficient and time-consuming even with the use of complex selection strategies (9–11). Intact CMV genomes can be maintained in *Escherichia coli* as bacterial artificial chromosomes

(BACs), which can be transfected into permissive cell types to reconstitute live virus (12). HCMV and several primate and rodent CMVs that are used as models for CMV disease, including GPCMV, have been successfully cloned as infectious BACs (13–16). The viral genome can be manipulated in *E. coli* using a variety of methodologies, including transposon mutagenesis and recombineering, to introduce site-specific or random changes (17, 18). BAC technology facilitated the characterization of many viral gene products, culminating in studies that have elucidated the requirement of many HCMV genes for replication in cell culture (19, 20). The genetic instability of CMV in tissue culture also complicates the generation of mutant viruses: serial passage of CMV in cell culture results in genetic changes that impact pathogenicity and

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inhibit nonhomologous end joining. To enrich for GPT-positive (GPT⁺) HDR recombinant virus in the culture supernatants, virus was passed once in medium containing 200 μ M mycophenolic acid and 25 μ M xanthine.

Evaluation of Cas9 cleavage and identification of mutant viruses. Virus stocks produced by transfection/infection (above) were evaluated using a GeneArt genomic cleavage detection kit (ThermoFisher) or a Guide-it mutation detection kit (Clontech). Primers were designed to amplify 500- to 750-bp products where the CRISPR site was in a noncentral location (see Table S2 in the supplemental material). For GeneArt assays, products were amplified from infected cell lysates, whereas for Guide-it assays, products were amplified directly from culture supernatants. PCR products were heated to 95°C, slow cooled to 37°C, and then incubated with the detection enzyme according to the manufacturer's specifications. In some experiments, where the Cas9 cleavage site overlapped a PvuI restriction enzyme site, the PCR product was incubated with PvuI (NEB) to detect loss of the PvuI site. Reaction mixtures were separated on 2% Tris-acetate-EDTA (TAE)-agarose gels and visualized with ethidium bromide. Predicted sizes of PCR products and their cleavage products are listed in Table S2 in the supplemental material.

Clonal mutant viruses were isolated by limiting dilution in 96-well plates. Seven to 10 days postinfection, RFP-positive candidate wells were passed to 12-well plates and a sample of cell or culture medium was PCR amplified using Terra PCR direct polymerase mix (Clontech) and the appropriate gene-specific primer set (see Table S2 in the supplemental material). For *GP133*, wild-type (WT) or indel mutations were predicted to generate ~582-bp products, while ~278- or 1,196-bp products indicated knockout or knock-in mutations, respectively. A single PCR product after limiting dilution was considered indicative of a clonal viral isolate. PCR products were cleaned using a QIAquick PCR purification kit (Qiagen) and Sanger sequenced by the University of Minnesota Genomics Center using gene-specific primers (sequences in bold in Table S2). Chromatographs were analyzed using Geneious (36). Isolates for which chromatographs contained mixed sequences, suggesting the presence of multiple viral variants, were not analyzed further. One milliliter of each P0 virus stock was mixed with 0.5 ml of 3 $\times$ sucrose phosphate buffer (0.65 M sucrose, 21 mM K₂HPO₄, and 11.5 mM KH₂PO₄) and stored frozen at -80°C (39).

Immunoblot analysis. GPL cells were infected with RFP GPCMV expressing hemagglutinin (HA)-tagged *GP133* at a multiplicity of infection (MOI) of 3. The culture medium was collected at 96 h p.i. and clarified by centrifugation at 170 $\times$ g for 10 min at 4°C. The remaining adherent, infected GPL cells, and uninfected control cells were scraped, pooled with the pelleted cells from the culture medium, and pelleted at 170 $\times$ g for 10 min, washed twice with PBS, and lysed in 2% SDS. Proteins were resolved on a Novex 12% Tris-glycine gel (ThermoFisher), transferred to a polyvinylidene difluoride membrane (Millipore), blocked with Amersham ECL prime blocking reagent (GE Healthcare), and probed with anti-HA (F-7; Santa Cruz Biotechnology), anti-2A (ABS31; Millipore), anti-GPCMV gB (monoclonal antibody 29-29), or anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (G041; ABM) primary antibodies in blocking buffer (40). Blots were developed using horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Cell Signaling Technologies) and Amersham ECL prime Western blotting detection reagent (GE Healthcare).

RESULTS

Mutation of *GP133* using CRISPR/Cas9-mediated gene editing. We first sought to test whether CRISPR/Cas9 could target the GPCMV genome and whether Cas9 expression and cleavage of viral DNA was deleterious to replication. Potential CRISPR target sites in the GPCMV genome that conformed to a 5'-GN(20)GG-3' motif were identified with the "Find CRISPR Sites" algorithm of Geneious (36). Nearly 10,000 potential CRISPR sites, targeting every predicted ORF in the GPCMV genome, were iden-

FIG 1 Targeted cleavage and repair of GPCMV. (A) Sequence of the *GP133* CRISPR site selected for pilot studies. The predicted Cas9 cleavage site and PvuI restriction site are shown. (B) Cells transfected with a gRNA expression plasmid targeting *GP133* or a nontargeting control (NT) were infected with GPCMV. An ~582-bp DNA fragment containing the CRISPR site was PCR amplified from infected cells (-) and subjected to either cleavage detection assay (+) or PvuI digestion (P). (C) Titers of virus-containing media collected 4 d p.i. from cells transfected with either NT or *GP133*-targeting gRNA expression plasmids. (D) Sequence alignment showing 11 unique *GP133* mutants compared to the CRISPR site in the WT virus. The type (I, insertion, or D, deletion) and size (in bp) of each mutation is shown, along with whether the *GP133* reading frame is maintained.

tified by the software. Individual CRISPR sites were scored by Geneious for potential off-target activity elsewhere in the viral genome. Sites with any predicted off-target activity were not used in this study.

A CRISPR site in *GP133*, one of the genes encoding the GPCMV gH/gL pentameric complex, was selected for the initial experiments (24). The predicted Cas9 cleavage site is located 47 bp from the 5' end of the ORF and overlaps a recognition site for the restriction endonuclease PvuI (Fig. 1A; see Table S1 in the supplemental material). In previous studies of the GPCMV pentameric

complex, *GP133* was not required for replication in fibroblasts (13, 41). Cas9 cleavage at the 5' end of *GP133* followed by repair of the resulting double-strand break by nonhomologous end joining (NHEJ) was expected to disrupt the *GP133* open reading frame in 2 out of 3 DNA repair events.

A 19-bp sequence targeting *GP133* (see *GP133* gRNA in Table S1 in the supplemental material) was cloned into a gRNA expression construct (gRNA plasmid) that contains a humanized *Streptococcus pyogenes* Cas9 expression cassette, a guide RNA (gRNA) regulated by the U6 promoter, and a puromycin-*N*-acetylase cassette. A nontargeting construct that has no predicted target in the GPCMV or guinea pig genome (see NT gRNA in Table S1 in the supplemental material) was also generated to investigate the effect of Cas9 expression on replication in the absence of targeted Cas9 cleavage of the GPCMV genome.

Confluent guinea pig lung fibroblasts (GPL cells) were transfected with these gRNA plasmids. In experiments where GPL cells were transfected with a GFP expression construct, approximately 50% of the cells were transfected. The cells were treated with puromycin beginning at 48 h after transfection. After 24 h of drug treatment (72 h posttransfection), the cells were infected with RFP GPCMV. The combination of transient transfection and puromycin treatment caused extensive cell death, which complicated an accurate calculation of MOI. Therefore, a dose of 2.6×10^4 PFU per cm^2 of transfected cells, equivalent to an MOI of 1 in untreated cells, was used for transfection/infection experiments described here.

Virus-containing media were collected at 96 h postinfection and passaged on GPL cells. DNA was extracted from the infected cells when extensive cytopathic effect and RFP expression were observed. A 582-bp fragment including *GP133* was PCR amplified from infected-cell DNA (Fig. 1B). To assay for Cas9 cleavage and repair by NHEJ, these DNA fragments were subjected to either a cleavage detection assay or PvuI restriction digestion. For the cleavage detection assay, the PCR product was melted and slow-cooled, enabling the formation of heteroduplexes if multiple DNA species were present, as would be the case if mutants had been generated. Cleavage of heteroduplexes by the detection enzyme was predicted to generate fragments 441 and 141 bp in size and indicated the presence of mutants generated by Cas9 cleavage and repair by NHEJ. Alternatively, PvuI digestion cleaves unedited *GP133*, but cleavage and repair by NHEJ is expected to destroy the PvuI recognition site, resulting in full-length PCR product after digestion. PCR product generated from cells infected with virus recovered from the NT gRNA plasmid transfection/infection was not digested by the cleavage detection enzyme and was cleaved by PvuI (Fig. 1B). In contrast, PCR products generated from cells infected with virus from the *GP133*-targeting gRNA transfection/infection were cleaved by the detection enzyme but not by PvuI (Fig. 1B). This suggests that cleavage and repair of *GP133* occurred and that virtually all of the recovered viral DNA was mutated at the predicted Cas9 cleavage site.

To determine whether Cas9 cleavage of the viral genome was detrimental to viral replication, titers of the virus-containing media recovered at 96 h postinfection from multiple gRNA plasmid transfection/infection experiments were determined (Fig. 1C). Across two experiments, a modest (~ 2 -fold; $P = 0.047$; 2-tailed t test) decrease in the recovery of virus from cells transfected with one of two *GP133*-targeting gRNA plasmids ($n = 3$ *GP133* 5'; $n = 2$ *GP133* 3') compared to virus recovered from cells transfected

with the nontargeting gRNA plasmid ($n = 5$) was observed. This observation suggests that cleavage of the GPCMV genome by Cas9 has a limited impact on viral replication.

To more accurately assess the efficiency of Cas9 cleavage and repair and to characterize individual viruses, GPCMV mutants were isolated by limiting dilution from virus-containing media collected from the *GP133* gRNA transfection/infection. *GP133* was PCR amplified from DNA extracted from 15 isolated plaques and Sanger sequenced (Fig. 1D). All 15 of these viruses had mutations in *GP133*, and 11 unique insertion or deletion (indel) mutations were identified. Ten of the 11 unique mutations were deletions centered on the predicted Cas9 cleavage site, and 9 of the 11 mutations introduced frame shifts that caused nonsense mutations in *GP133*, while 2 of the 11 mutations maintained the reading frame. Together, these experiments demonstrated that CRISPR/Cas9 cleavage and repair of the GPCMV genome occurred and that this mutagenesis strategy generated gene-disrupting indel mutations.

Deletion of *GP133* by CRISPR/Cas9 editing. Whether CRISPR/Cas9 editing can deliberately delete larger portions of the viral genome was also investigated. Using the transfection/infection strategy described above, GPL cells were cotransfected with either a single gRNA expression construct targeting the 5' or 3' end of *GP133* and an irrelevant plasmid or a mix of the two gRNA plasmids (see Table S1 in the supplemental material). At 96 h postinfection, virus-containing media were subjected to direct PCR using *GP133*-specific primers (Fig. 2A). Virus-containing medium from each transfection/infection with a single gRNA plasmid generated an ~ 582 -bp PCR product that corresponded to unedited *GP133* or to viruses with small indels at the mutation site. In contrast, the PCR product generated from virus-containing medium from cells transfected with the two gRNA plasmids also contained a 278-bp product that corresponded to the deletion of sequence between the two Cas9 cleavage sites in *GP133*. Sixteen plaques were isolated by serial dilution of the virus-containing medium from the 5' and 3' *GP133* gRNA cotransfection. When the *GP133* locus was PCR amplified from these GPCMV isolates, a single ~ 278 -bp fragment was detected from 8 of the 16 plaques (Fig. 2B). In addition to these eight candidate *GP133* deletion viruses, a single ~ 582 -bp fragment was amplified from four of the plaques. These viruses likely represented either isolates with indel mutations at each *GP133* Cas9 cleavage site or wild-type virus but were not characterized further. The remaining four plaques yielded multiple PCR products and were likely a mixed population of viruses. The eight candidate *GP133* deletion viruses were Sanger sequenced (Fig. 2C). Four unique *GP133* deletions were recovered; each virus contained a deletion in *GP133* of between 304 and 311 bp in size. The most frequently recovered mutation (5 of 8 plaques) was a 304-bp deletion that corresponded to direct ligation of the DNA ends that result from cleavage at the predicted Cas9 target sites. These experiments demonstrated that cotransfection with two gRNA plasmids can be used to delete specific large pieces of the viral genome.

Epitope tagging of *GP133* by homology-directed repair. Double-strand breaks introduced by CRISPR/Cas9 editing can be repaired by homology-directed repair (HDR) if a suitable homologous recombination template (HRT) is available in the cell. An HRT was cloned into a gRNA plasmid that targeted the 3' end of *GP133* (Fig. 3A). The *GP133* 3' CRISPR site of the HRT was mutated to prevent Cas9 recognition of the site while retaining the

FIG 2 Knockout of *GP133* by CRISPR/Cas9 editing. (A) *GP133* was amplified from virus-containing media collected from cells transfected with gRNA expression constructs targeting the 5' or 3' end of *GP133* plus a GFP expression cassette or a mix of the two gRNA expression constructs and visualized by electrophoresis. (B) *GP133* was amplified from plaques isolated by limiting dilution using PCR. Full-length *GP133* was expected to produce a 582-bp fragment, while deletion of sequence between the Cas9 cleavage sites would result in a 278-bp PCR product. (C) Candidate *GP133* deletion viruses, indicated by asterisked lanes in panel B, were Sanger sequenced. The unique viruses were aligned to the WT sequence. The CRISPR and predicted Cas9 cleavage sites are shown above the alignment. Due to space limitations, 281 bp of sequence between the CRISPR sites in the wild-type sequence is not shown in the figure. The most frequently recovered mutant is indicated by an asterisk.

normal *GP133* amino acid sequence. The 3' end of *GP133* in the HRT was tagged by sequences encoding an HA epitope followed by a "self-cleaving" 2A peptide and coding sequences for GPT (37, 38). Thus, virus generated by HDR using this construct was predicted to express an HA-tagged version of *GP133* and GPT as a

multicistronic transcript. Flanking 600-bp homologies were also included in the HRT.

gRNA plasmids targeting the *GP133* 3' end with and without the HRT were used in a transfection/infection experiment as described above. Double-strand DNA breaks in mammalian cells are

FIG 3 Epitope-tagging of *GP133* by homology-directed repair. (A) Diagram of the homology repair template (HRT) used to introduce the HA-2A-GPT tag to the 3' end of *GP133*. Modifications to the *GP133* coding sequence (CDS) were as follows: amino acids overlapping the CRISPR site were codon optimized (black arrow) and sequences encoding a 3' HA-tag, a glycine-serine-glycine (GSG) spacer, a 2AT peptide, and a flippase recognition target (FRT)-flanked GPT were added. (B) PCR amplification of passaged virus-containing media from transfection/infection experiments using gRNA plasmids targeting the 3' end of *GP133*, with or without the HRT. The initial transfection/infections were carried out in the presence or absence of the DNA ligase IV inhibitor SCR7, and the virus was passaged with or without GPT selection. (C) PCR amplification of *GP133* from viruses isolated either immediately after transfection/infection or after a single passage in media containing mycophenolic acid xanthine to enrich for recombinant viruses. (D) Immunoblots of cell lysates from mock-infected GPL cells and cells infected with GPCMV *GP133*-HA-2A-GPT or its parental virus, RFP GPCMV. Primary antibodies against the HA tag, 2A peptide, GPCMV gB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used.

repaired by either NHEJ or HDR, but repair by NHEJ is generally more frequent (42). SCR7, an inhibitor of NHEJ that blocks DNA ligase IV activity, increased the rate of recovery of HDR mutants from CRISPR/Cas9-editing experiments in mammalian cells (43). To investigate whether SCR7 could also increase the recovery of GPCMV mutants repaired by HDR, a subset of transfections was treated with 1 μ M SCR7 at the time of puromycin selection. Virus-containing media were collected 96 h postinfection and passaged on GPL cells. Medium containing mycophenolic acid and xanthine was added to half of these infections with passaged virus with the aim of using metabolic selection to enrich HDR mutant viruses expressing GPT. *GP133* was amplified directly from the passaged-virus-containing media (Fig. 3B). In the cells transfected with gRNA plasmids without the HRT, only an ~582-bp product was detected, corresponding to either unedited *GP133* or *GP133* repaired by NHEJ. Multiple amplification products were detected from passaged-virus-containing media when the gRNA plasmid that included the HRT was used for the initial transfection/infection. These amplicons included an 1,196-bp fragment that corresponded to epitope-tagged *GP133* mutants generated by HDR. The ratios of 582- to 1,196-bp PCR products varied, with the highest levels of 1,196-bp fragments recovered after GPT selection (Fig. 3B).

After isolating clonal viruses by limiting dilution, the frequency of HDR of GPCMV was estimated. An 1,196-bp fragment was amplified from 1 of the 12 plaques isolated from virus-containing media collected immediately after transfection/infection with the gRNA plasmid that included the HRT (Fig. 3C). The presence of SCR7 during the transfection/infection did not increase the frequency of these apparent HDR mutant viruses. A single passage in the presence of mycophenolic acid and xanthine did enrich HDR mutants, as 1,196-bp fragments were amplified from 5 of the 12 isolated viruses. Additional PCRs using primers external to the HRT sequence confirmed that the epitope tag was inserted at the 3' end of *GP133* (data not shown). Sanger sequencing of three apparent *GP133* HDR mutant viruses further verified knock-in of the HA-2A-GPT tag (data not shown). GPL cells were infected with either tagged virus or its parental strain, and lysate from infected cells was subjected to immunoblot analysis. HA-tagged *GP133* was predicted to have a mass of 17.8 kDa. A protein of this size was detected in cells infected with the *GP133* HA-tagged virus when antibodies against either the HA tag or 2A peptide were used (Fig. 3D). These experiments demonstrate that foreign DNA can be introduced to the GPCMV genome by HDR after Cas9 cleavage.

CRISPR/Cas9-mediated editing as a strategy to screen viral gene function. A panel of gRNA expression constructs was engineered to better understand the efficiency and versatility of the CRISPR/Cas9-based mutagenesis strategy. gRNA plasmids were cloned with the intent of disrupting every member of the US22 gene family in the GPCMV genome. The US22 gene family is unique to the betaherpesviruses; each sequenced CMV encodes approximately 12 members of the family (44, 45). Global deletion studies with HCMV found that disruption of individual members of the US22 family either had a negligible effect on replication in cell culture or resulted in attenuated viruses (19, 20). In murine CMV (MCMV), only the US22 family genes *m142* and *m143* were essential for replication (46). We have previously characterized GPCMV Δ *gp145*, a severely attenuated virus with a defect in the viral response to the antiviral protein kinase R (PKR) pathway,

FIG 4 CRISPR/Cas9 editing of US22 family genes. (A) GPL cells were transfected with gRNA expression constructs targeting the indicated genes and infected with GPCMV. Targeted genes were amplified (–) directly from virus-containing media at 96 h p.i. and subjected to cleavage detection analysis (+). The predicted sizes for each cleavage detection reaction are outlined in Table S2 in the supplemental material. (B) Multistep growth curve analysis of GPCMV with targeted disruptions in *gp144* or *gp145* compared to the parental strain (WT). GPL cells were infected at an MOI of 0.01, virus-containing media were collected, and titers were determined at the indicated time points. Data points represent mean titer of duplicate infections.

and have observed that several GPCMV US22 family proteins have nucleic acid binding activity (47, 48).

Fourteen gRNA plasmids targeting US22 family members were cloned (see Table S1 in the supplemental material). An additional plasmid targeting the multiple cloning site upstream of the RFP gene in RFP GPCMV (MCS) was also engineered. GPL cells were transfected with these plasmids and infected with RFP GPCMV as described above. Ninety-six hours postinfection, each targeted gene was PCR amplified directly from this virus-containing media and subjected to cleavage detection analysis (Fig. 4A). While the apparent efficiency of editing varied from construct to construct, cleaved PCR products were observed in all cases. Low levels of cleavage were observed when *gp144*, *gp145*, and *gp146* were targeted, perhaps suggesting less efficient cleavage and repair of these genes. However, this cleavage analysis measures PCR product heterogeneity rather than specifically assaying for CRISPR/Cas9 cleavage and repair. The presence of an uncleaved PCR product indicates the presence of DNA homoduplexes after melting and

TABLE 1 Summary of GPCMV CRISPR-Cas9 mutagenesis

GPCMV gene	Homolog in HCMV ^a	No. of plaques	No. of WT plaques	Mutant frequency	No. of unique mutants	No. of NS ^b mutants	Frequency of NS ^b mutants	No. of TR recombinants ^c
<i>gp3</i>	<i>US22</i>	10	1	0.90	6	5	0.83	0
<i>GP23</i>	<i>UL23</i>	10	2	0.80	8	6	0.75	0
<i>GP24</i>	<i>UL24</i>	11	3	0.73	7	4	0.57	1
<i>GP28</i>	<i>UL28</i>	12	2	0.83	7	6	0.86	0
<i>GP28.1</i>	<i>UL29</i>	11	1	0.91	9	7	0.78	1
<i>GP29</i>	<i>UL29</i>	12	0	1.00	8	7	0.88	0
<i>GP29.1</i>	<i>UL29</i>	12	2	0.83	7	6	0.86	0
<i>GP133</i>	<i>UL131</i>	20	0	1.00	12	10	0.83	0
<i>gp139</i>	<i>US22</i>	10	0	1.00	9	7	0.78	0
<i>gp141</i>	<i>US23</i>	8	0	1.00	7	7	1.00	0
<i>gp142</i>	<i>US24</i>	12	0	1.00	9	5	0.56	1
<i>gp143</i>	<i>US22</i>	11	0	1.00	7	6	0.86	0
<i>gp144</i>	<i>US26</i>	19	10	0.47	7	2	0.29	2
<i>gp145</i>	<i>TRS1/IRS1</i>	11	1	0.91	9	2	0.22	0
<i>gp146</i>	<i>TRS1/IRS1</i>	12	1	0.92	8	6	0.75	0
MCS	N/A	16	5	0.69	4	3	0.75	0
Total		197	28	0.86	124	89	0.72	5

^a Based on homology to HCMV genes (49, 50). N/A, not applicable.

^b NS, nonsense.

^c Mutants with apparent recombination between the Cas9 cleavage site and the terminal repeat (TR) sequences.

reannealing; plaque isolation and sequencing of the predicted Cas9 cleavage site is necessary to accurately assess the efficiency of mutagenesis.

In this study, a total of 197 plaques were isolated and sequenced, the results of which are summarized in Table 1. One hundred sixty-nine of 197 isolates contained mutated GPCMV sequences, suggesting an overall mutant recovery rate of 86%. Double-strand DNA break repair by NHEJ is expected to result in a nonsense mutation in 2 of 3 unique repair events. Nonsense mutants were recovered at or above this frequency for most genes targeted in this study; over 70% of unique viruses were nonsense mutants. Two targeted genes did not conform to this expectation: *gp144* and *gp145*. Fewer than 30% of the unique indel mutations in *gp144* and *gp145* resulted in nonsense mutations. Previous studies of *gp145* function have revealed that a knockout virus is attenuated (47). Multistep growth curve analysis of both *gp144* and *gp145* nonsense mutants found that the viruses were also attenuated (Fig. 4B). The replication defect of the *gp145* nonsense mutant was comparable to that of the previously characterized whole-gene knockout virus (47). This leads us to conclude that in-frame indel mutations and wild-type viruses can outcompete nonsense mutants when disrupting a targeted viral gene is deleterious to replication.

The size and type of the 124 unique GPCMV indel mutations generated by CRISPR/Cas9 editing was further characterized. Figure 5 summarizes the total sequence gain or loss by indel mutants at their Cas9 cleavage sites. Deletion viruses (88 of 124 isolates) were more common than insertion viruses, and most deletions were small (68 of 124 viruses had 1- to 5-bp deletions). Large, >5-bp insertion or deletion viruses were not uncommon (37 of 124 mutants). Many viruses had some combination of nucleotide deletion and insertion at the Cas9 cleavage site (for examples, see Fig. 1C). A number of very large (>50 bp) insertion viruses were recovered. Because relatively small PCR products were sequenced in this analysis, it is possible that viruses with large deletions at the cleav-

age site never PCR amplified and that these viruses were missed by our analysis. Five viruses with apparent recombination between GPCMV terminal repeat regions (TRR) and the Cas9 cleavage sites were also identified but were not further characterized. In conclusion, CRISPR/Cas9 mutagenesis is a versatile and efficient technique that can be used to introduce targeted changes into many GPCMV genes.

DISCUSSION

We report the first use of CRISPR/Cas9-mediated gene editing to introduce targeted mutations into the GPCMV genome. CRISPR/Cas9 gene editing has recently been employed to manipulate the genomes of a number of large DNA viruses, and this study represents the first application of the technology to a cytomegalovirus (28, 30–33). Using a simple transfection/infection methodology, we demonstrate that CRISPR/Cas9 editing can efficiently generate nonsense, knockout, and knock-in GPCMV mutants.

One potential limitation of this technology is off-target activity

FIG 5 Summary of unique GPCMV CRISPR/Cas9 mutants recovered. The net size and number of insertion or deletion mutants recovered in this study is shown. Results for mutations that maintain the open reading frame are shown as hatched columns. The number of terminal repeat recombinant (TRR) viruses is also shown.

of Cas9, which can tolerate up to a 5-bp mismatch between a guide RNA and a DNA sequence for its cleavage activity (51, 52). Algorithms used to identify potential CRISPR sites can also assess potential off-target activity by identifying similar DNA sequences in a genome. Viral genomes are orders of magnitude smaller than mammalian genomes, and only a fraction of CRISPR sites have any level of predicted off-target activity elsewhere in the viral genome. Off-target mutations have not been detected by deep sequencing of the genomes of CRISPR/Cas9-edited viruses in previous studies (28, 33). Restriction fragment length profiling is frequently used to check the genome architecture of BAC-derived viruses, but whole-genome sequencing of recombinant CMVs to identify mutations is not yet routine. In this study, we used predictive algorithms to exclude potential CRISPR sites with homologous sequences elsewhere in the genome, but have not yet carried out whole-genome sequencing to assess the overall frequency of off-target mutation.

It is also possible that off-target cleavage of the host genome by Cas9 could be deleterious to viral replication. We did not actively evaluate whether gRNAs designed to target GPCMV could also cleave the guinea pig genome. However, GPL cells need to support only a single round of GPCMV replication for CRISPR/Cas9 mutagenesis to succeed, and viral stocks are amplified in normal cells. Having edited GPCMV with 17 unique gRNA targeting constructs, we encountered only one difficulty: recovering gene-disrupting mutations in *gp144* and *gp145*. In both cases, these genes appear to augment GPCMV replication, and their disruption results in growth-attenuated mutant viruses (47).

In a recent CRISPR/Cas9-editing study in adenovirus, the speed of viral replication appeared to outpace the mammalian DNA damage repair machinery, limiting the efficiency of CRISPR/Cas9 editing (28). We observed only a modest decrease in the recovery of progeny virus from cells transfected with gRNAs that cleaved *GP133* compared to that from cells expressing a non-targeting gRNA (Fig. 1C). The slow replicative cycle of CMV may make the virus well-suited for CRISPR/Cas9 mutagenesis: in single-step growth curve analysis of GPCMV, progeny virus is not detected until 36 h p.i., with maximal titers achieved at 48 to 72 h p.i. (13, 14). We suspect that Cas9 cleavage of the viral genome may have a greater impact on replication at earlier time points or when CRISPR sites located in augmenting or essential genes are cleaved by Cas9. For the described transfection/infection experiments, we used a high (~1) MOI based on the observation that CRISPR/Cas9 editing of adenovirus was not efficient during low-MOI infections (28). However, mutant GPCMV has also been recovered in other experiments when 100-fold less virus was used for the infection (data not shown), suggesting that our methodology is not dependent on high-MOI infection.

BAC-based technology is currently the most frequently used CMV mutagenesis strategy. Some viral sequences are toxic when introduced to bacteria, and this toxicity is most noticeable when viral DNA is maintained on high-copy-number plasmids (53). Maintaining the CMV genome as a low-copy-number BAC overcomes this potential problem, but spontaneous mutations have been documented to occur during BAC maintenance (54). These mutations may be due to unnatural selective pressures encountered by the viral DNA in *E. coli* or simply be rare spontaneous events that result from the numerous replicative cycles that the large viral genome undergoes as BACs are grown and manipulated. Reconstituting virus from a BAC can be challenging: the

large viral genome can easily be sheared during manipulation, and human fibroblasts are often difficult to transfect. However, virtually any mutation can be introduced to a CMV BAC, regardless of an impact on viral fitness, and many potentially lethal mutations can be rescued by reconstituting the virus on a *trans*-complementing cell line.

CRISPR/Cas9 mutagenesis is a technically simple alternative to BAC recombineering that eliminates the need for a BAC intermediate. This methodology could be used to genetically manipulate low-passage clinical isolates. A working stock of mutant virus can be generated by CRISPR/Cas9 mutagenesis in as few as three passages. It may also be possible to manipulate CMV genomes in nonfibroblast cell types, which may reduce the risk for common viral adaptations to cell culture, such as the loss or mutation of the gH/gL pentameric complex (21, 22). While questions remain about potential off-target activity by Cas9 on the viral genome, indel mutations at a Cas9 cleavage site are generated by transfection of a single gRNA at a high frequency. NHEJ can also be used to generate defined knockout viruses by eliminating the sequence between two Cas9 cleavage sites. Simple modifications to the described transfection/infection workflow can also utilize HDR to knock-in specific sequences and allow more precise editing, albeit at lower frequencies. Future studies will utilize next-generation whole-genome sequencing to directly compare the rates of spontaneous or off-target mutations between BAC- and CRISPR/Cas9-derived GPCMV mutants.

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REFERENCES

1. Mocarski ES, Jr, Shenk T, Griffiths PD, Pass RF. 2013. Cytomegaloviruses, p 1960–2014. In Knipe DM, Howley PM, Cohen JL, Griffin DE, Lamb RA, Martin MA, Racaniello VR, Roizman B (ed), *Fields virology*, 6th ed, vol 2. Lippincott Williams & Wilkins, Philadelphia, PA.
2. Kenneson A, Cannon MJ. 2007. Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection. *Rev Med Virol* 17:253–276. <http://dx.doi.org/10.1002/rmv.535>.
3. Chee MS, Bankier AT, Beck S, Bohni R, Brown CM, Cerny R, Horsnell T, Hutchison CA, III, Kouzarides T, Martignetti JA, Preddie E, Satchwell SC, Tomlinson P, Weston KM, Barrell BG. 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr Top Microbiol Immunol* 154:125–169.
4. Stern-Ginossar N, Weisburd B, Michalski A, Le VT, Hein MY, Huang SX, Ma M, Shen B, Qian SB, Hengel H, Mann M, Ingolia NT, Weissman JS. 2012. Decoding human cytomegalovirus. *Science* 338:1088–1093. <http://dx.doi.org/10.1126/science.1227919>.
5. Johnson KP, Connor WS. 1979. Guinea pig cytomegalovirus: transplacental transmission. *Arch Virol* 59:263–267. <http://dx.doi.org/10.1007/BF01317422>.
6. Auerbach MR, Yan D, Vij R, Hongo JA, Nakamura G, Vernes JM, Meng YG, Lein S, Chan P, Ross J, Carano R, Deng R, Lewin-Koh N, Xu M,

- Feierbach B. 2014. A neutralizing anti-gH/gL monoclonal antibody is protective in the guinea pig model of congenital CMV infection. *PLoS Pathog* 10:e1004060. <http://dx.doi.org/10.1371/journal.ppat.1004060>.
7. Kumar ML, Nankervis GA. 1978. Experimental congenital infection with cytomegalovirus: a guinea pig model. *J Infect Dis* 138:650–654. <http://dx.doi.org/10.1093/infdis/138.5.650>.
8. Manning WC, Mocarski ES. 1988. Insertional mutagenesis of the murine cytomegalovirus genome: one prominent alpha gene (ie2) is dispensable for growth. *Virology* 167:477–484. [http://dx.doi.org/10.1016/S0042-6822\(88\)90110-9](http://dx.doi.org/10.1016/S0042-6822(88)90110-9), [http://dx.doi.org/10.1016/0042-6822\(88\)90110-9](http://dx.doi.org/10.1016/0042-6822(88)90110-9).
9. Vieira J, Farrell HE, Rawlinson WD, Mocarski ES. 1994. Genes in the HindIII J fragment of the murine cytomegalovirus genome are dispensable for growth in cultured cells: insertion mutagenesis with a lacZ/gpt cassette. *J Virol* 68:4837–4846.
10. Wolff D, Jahn G, Plachter B. 1993. Generation and effective enrichment of selectable human cytomegalovirus mutants using site-directed insertion of the neo gene. *Gene* 130:167–173. [http://dx.doi.org/10.1016/0378-1119\(93\)90416-Z](http://dx.doi.org/10.1016/0378-1119(93)90416-Z).
11. Greaves RF, Brown JM, Vieira J, Mocarski ES. 1995. Selectable insertion and deletion mutagenesis of the human cytomegalovirus genome using the *Escherichia coli* guanosine phosphoribosyl transferase (gpt) gene. *J Gen Virol* 76:2151–2160. <http://dx.doi.org/10.1099/0022-1317-76-9-2151>.
12. Messerle M, Crnkovic I, Hammerschmidt W, Ziegler H, Koszinowski UH. 1997. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. *Proc Natl Acad Sci U S A* 94:14759–14763. <http://dx.doi.org/10.1073/pnas.94.26.14759>.
13. Cui X, McGregor A, Schleiss MR, McVoy MA. 2008. Cloning the complete guinea pig cytomegalovirus genome as an infectious bacterial artificial chromosome with excisable origin of replication. *J Virol Methods* 149:231–239. <http://dx.doi.org/10.1016/j.jviromet.2008.01.031>.
14. McGregor A, Schleiss MR. 2001. Molecular cloning of the guinea pig cytomegalovirus (GPCMV) genome as an infectious bacterial artificial chromosome (BAC) in *Escherichia coli*. *Mol Genet Metab* 72:15–26. <http://dx.doi.org/10.1006/mgme.2000.3102>.
15. Borst EM, Hahn G, Koszinowski UH, Messerle M. 1999. Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in *Escherichia coli*: a new approach for construction of HCMV mutants. *J Virol* 73:8320–8329.
16. Chang WL, Barry PA. 2003. Cloning of the full-length rhesus cytomegalovirus genome as an infectious and self-excisable bacterial artificial chromosome for analysis of viral pathogenesis. *J Virol* 77:5073–5083. <http://dx.doi.org/10.1128/JVI.77.9.5073-5083.2003>.
17. Brune W, Menard C, Hobom U, Odenbreit S, Messerle M, Koszinowski UH. 1999. Rapid identification of essential and nonessential herpesvirus genes by direct transposon mutagenesis. *Nat Biotechnol* 17:360–364. <http://dx.doi.org/10.1038/7914>.
18. Warden C, Tang Q, Zhu H. 2011. Herpesvirus BACs: past, present, and future. *J Biomed Biotechnol* 2011:124595.
19. Dunn W, Chou C, Li H, Hai R, Patterson D, Stolz V, Zhu H, Liu F. 2003. Functional profiling of a human cytomegalovirus genome. *Proc Natl Acad Sci U S A* 100:14223–14228. <http://dx.doi.org/10.1073/pnas.2334032100>.
20. Yu D, Silva MC, Shenk T. 2003. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. *Proc Natl Acad Sci U S A* 100:12396–12401. <http://dx.doi.org/10.1073/pnas.1635160100>.
21. Cha TA, Tom E, Kemble GW, Duke GM, Mocarski ES, Spaete RR. 1996. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. *J Virol* 70:78–83.
22. Dargan DJ, Douglas E, Cunningham C, Jamieson F, Stanton RJ, Baluchova K, McSharry BP, Tomasec P, Emery VC, Percivalle E, Sarasini A, Gerna G, Wilkinson GW, Davison AJ. 2010. Sequential mutations associated with adaptation of human cytomegalovirus to growth in cell culture. *J Gen Virol* 91:1535–1546. <http://dx.doi.org/10.1099/vir.0.018994-0>.
23. Ryckman BJ, Rainish BL, Chase MC, Borton JA, Nelson JA, Jarvis MA, Johnson DC. 2008. Characterization of the human cytomegalovirus gH/gL/UL128–131 complex that mediates entry into epithelial and endothelial cells. *J Virol* 82:60–70. <http://dx.doi.org/10.1128/JVI.01910-07>.
24. Auerbach M, Yan D, Fouts A, Xu M, Estevez A, Austin CD, Bazan F, Feierbach B. 2013. Characterization of the guinea pig CMV gH/gL/GP129/GP131/GP133 complex in infection and spread. *Virology* 441:75–84. <http://dx.doi.org/10.1016/j.virol.2013.03.008>.
25. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337:816–821. <http://dx.doi.org/10.1126/science.1225829>.
26. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. 2013. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8:2281–2308. <http://dx.doi.org/10.1038/nprot.2013.143>.
27. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. 2013. RNA-guided human genome engineering via Cas9. *Science* 339:823–826. <http://dx.doi.org/10.1126/science.1232033>.
28. Bi Y, Sun L, Gao D, Ding C, Li Z, Li Y, Cun W, Li Q. 2014. High-efficiency targeted editing of large viral genomes by RNA-guided nucleases. *PLoS Pathog* 10:e1004090. <http://dx.doi.org/10.1371/journal.ppat.1004090>.
29. Russell TA, Stefanovic T, Tschärke DC. 2015. Engineering herpes simplex viruses by infection-transfection methods including recombination site targeting by CRISPR/Cas9 nucleases. *J Virol Methods* 213:18–25. <http://dx.doi.org/10.1016/j.jviromet.2014.11.009>.
30. Suenaga T, Kohyama M, Hirayasu K, Arase H. 2014. Engineering large viral DNA genomes using the CRISPR-Cas9 system. *Microbiol Immunol* 58:513–522. <http://dx.doi.org/10.1111/1348-0421.12180>.
31. Xu A, Qin C, Lang Y, Wang M, Lin M, Li C, Zhang R, Tang J. 2015. A simple and rapid approach to manipulate pseudorabies virus genome by CRISPR/Cas9 system. *Biotechnol Lett* 37:1265–1272. <http://dx.doi.org/10.1007/s10529-015-1796-2>.
32. Yuan M, Zhang W, Wang J, Al Yaghchi C, Ahmed J, Chard L, Lemoine NR, Wang Y. 2015. Efficiently editing the vaccinia virus genome by using the CRISPR-Cas9 system. *J Virol* 89:5176–5179. <http://dx.doi.org/10.1128/JVI.00339-15>.
33. Yuen KS, Chan CP, Wong NH, Ho CH, Ho TH, Lei T, Deng W, Tsao SW, Chen H, Kok KH, Jin DY. 2015. CRISPR/Cas9-mediated genome editing of Epstein-Barr virus in human cells. *J Gen Virol* 96:626–636. <http://dx.doi.org/10.1099/jgv.0.000012>.
34. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. 2005. Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res* 33:e36. <http://dx.doi.org/10.1093/nar/gni035>.
35. Yang D, Alam Z, Cui X, Chen M, Sherrod CJ, McVoy MA, Schleiss MR, Dittmer DP. 2014. Complete genome sequence of cell culture-attenuated guinea pig cytomegalovirus cloned as an infectious bacterial artificial chromosome. *Genome Announc* 2(5):e00928-14.
36. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647–1649. <http://dx.doi.org/10.1093/bioinformatics/bts199>.
37. Falkner FG, Moss B. 1988. *Escherichia coli* gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. *J Virol* 62:1849–1854.
38. Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, Vignali DA. 2004. Correction of multi-gene deficiency in vivo using a single ‘self-cleaving’ 2A peptide-based retroviral vector. *Nat Biotechnol* 22:589–594. <http://dx.doi.org/10.1038/nbt957>.
39. Howell CL, Miller MJ. 1983. Effect of sucrose phosphate and sorbitol on infectivity of enveloped viruses during storage. *J Clin Microbiol* 18:658–662.
40. Britt WJ, Harrison C. 1994. Identification of an abundant disulfide-linked complex of glycoproteins in the envelope of guinea pig cytomegalovirus. *Virology* 201:294–302. <http://dx.doi.org/10.1006/viro.1994.1294>.
41. Gnanandarajah JS, Gillis PA, Hernandez-Alvarado N, Higgins L, Markowski TW, Sung H, Lumley S, Schleiss MR. 2014. Identification by mass spectrometry and immune response analysis of guinea pig cytomegalovirus (GPCMV) pentameric complex proteins GP129, 131 and 133. *Viruses* 6:727–751. <http://dx.doi.org/10.3390/v6020727>.
42. Mao Z, Bozzella M, Seluanov A, Gorbunova V. 2008. DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells. *Cell Cycle* 7:2902–2906. <http://dx.doi.org/10.4161/cc.7.18.6679>.
43. Chu VT, Weber T, Wefers B, Wurst W, Sander S, Rajewsky K, Kuhn R. 2015. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nat Biotechnol* 33:543–548. <http://dx.doi.org/10.1038/nbt.3198>.
44. Weston K, Barrell BG. 1986. Sequence of the short unique region, short

- repeats, and part of the long repeats of human cytomegalovirus. *J Mol Biol* 192:177–208. [http://dx.doi.org/10.1016/0022-2836\(86\)90359-1](http://dx.doi.org/10.1016/0022-2836(86)90359-1).
45. Davison AJ, Bhella D. 2007. Chapter 14. Comparative genome and virion structure. In Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, Yamanishi K (ed), *Human herpesviruses: biology, therapy, and immunoprophylaxis*. Cambridge University Press, Cambridge, UK.
 46. Ménard C, Wagner M, Ruzsics Z, Holak K, Brune W, Campbell AE, Koszinowski UH. 2003. Role of murine cytomegalovirus US22 gene family members in replication in macrophages. *J Virol* 77:5557–5570. <http://dx.doi.org/10.1128/JVI.77.10.5557-5570.2003>.
 47. Schleiss MR, Bierle CJ, Swanson EC, McVoy MA, Wang JB, Al-Mahdi Z, Geballe AP. 2015. Vaccination with a live attenuated cytomegalovirus devoid of a protein kinase R inhibitory gene results in reduced maternal viremia and improved pregnancy outcome in a guinea pig congenital infection model. *J Virol* 89:9727–9738. <http://dx.doi.org/10.1128/JVI.01419-15>.
 48. Bierle CJ, Schleiss MR, Geballe AP. 2012. Antagonism of the protein kinase R pathway by the guinea pig cytomegalovirus US22-family gene gp145. *Virology* 433:157–166. <http://dx.doi.org/10.1016/j.virol.2012.08.005>.
 49. Schleiss MR, McGregor A, Choi KY, Date SV, Cui X, McVoy MA. 2008. Analysis of the nucleotide sequence of the guinea pig cytomegalovirus (GPCMV) genome. *Virology* 375:139. <http://dx.doi.org/10.1016/j.virol.2008.03.015>.
 50. Kanai K, Yamada S, Yamamoto Y, Fukui Y, Kurane I, Inoue N. 2011. Re-evaluation of the genome sequence of guinea pig cytomegalovirus. *J Gen Virol* 92:1005–1020. <http://dx.doi.org/10.1099/vir.0.027789-0>.
 51. Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD. 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* 31:822–826. <http://dx.doi.org/10.1038/nbt.2623>.
 52. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F. 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* 31:827–832. <http://dx.doi.org/10.1038/nbt.2647>.
 53. Tischer BK, Kaufer BB. 2012. Viral bacterial artificial chromosomes: generation, mutagenesis, and removal of mini-F sequences. *J Biomed Biotechnol* 2012:472537.
 54. Murrell I, Wilkie GS, Davison AJ, Statkute E, Fielding C, Tomasec P, Wilkinson GW, Stanton RJ. 2016. Genetic stability of bacterial artificial chromosome-derived human cytomegalovirus during culture in vitro. *J Virol* 90:3929–3943. <http://dx.doi.org/10.1128/JVI.02858-15>.