Cloning of the Full-Length Rhesus Cytomegalovirus Genome as an Infectious and Self-Excisable Bacterial Artificial Chromosome for Analysis of Viral Pathogenesis

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Rigorous investigation of many functions encoded by cytomegaloviruses (CMVs) requires analysis in the context of virus-host interactions. To facilitate the construction of rhesus CMV (RhCMV) mutants for in vivo studies, a bacterial artificial chromosome (BAC) containing an enhanced green fluorescent protein (EGFP) cassette was engineered into the intergenic region between unique short 1 (US1) and US2 of the full-length viral genome by Cre/lox-mediated recombination. Infectious virions were recovered from rhesus fibroblasts transfected with pRhCMV/BAC-EGFP. However, peak virus yields of cells infected with reconstituted progeny were 10-fold lower than wild-type RhCMV, suggesting that inclusion of the 9-kb BAC sequence impeded viral replication. Accordingly, pRhCMV/BAC-EGFP was further modified to enable efficient excision of the BAC vector from the viral genome after transfection into mammalian cells. Allelic exchange was performed in bacteria to substitute the cre recombinase gene for egfp. Transfection of rhesus fibroblasts with pRhCMV/BAC-Cre resulted in a pure progeny population lacking the vector backbone without the need of further manipulation. The genomic structure of the BAC-reconstituted virus, RhCMV-loxP(r), was identical to that of wild-type RhCMV except for the residual loxP site. The presence of the loxP sequence did not alter the expression profiles of neighboring open reading frames. In addition, RhCMV-loxP(r) replicated with wild-type kinetics both in tissue culture and seronegative immunocompetent macaques. Restriction analysis of the viral genome present within individual BAC clones and virions revealed that (i) RhCMV exhibits a simple genome structure and that (ii) there is a variable number of a 750-bp iterative sequence present at the S terminus.

Infection of rhesus macaques (Macaca mulatta) with rhesus cytomegalovirus (RhCMV) is a relevant model for the study of human CMV (HCMV) pathogenesis. In addition to the fact that their hosts share strong developmental, physiological, and evolutionary similarities, HCMV and RhCMV exhibit essentially colinear genomes (D. G. Anders and S. Wong, unpublished data) and show very similar clinical sequelae in their hosts. Both viruses have a high seroprevalence, establish a lifelong asymptomatic persistence in the immunocompetent hosts (4, 8, 21), and cause severe disease in immunologically immature or immunocompromised individuals (5, 8, 22, 37, 48). Genetic analysis into the mechanisms of CMV persistence and pathogenesis is most relevant in vivo where interactions between virus and host cells take place. This is particularly true for the viral immunomodulatory genes that may not manifest an in vitro phenotype. This type of approach requires the ability to efficiently manipulate the viral genome to enable functional analysis of specific viral genes.

Due to the large genome size and slow replication kinetics of CMV, it is difficult to genetically engineer the CMV genomes through homologous recombination in mammalian cells. Recent advances of cloning and alteration of large DNA fragments in Escherichia coli have greatly facilitated the progression of CMV genomics (reviewed in references 10 and 50). Since the first description of using the bacterial artificial chro-

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system reduces the potential for random deletion of viral sequences and attenuation of reconstituted progeny.

In the present study, the construction of a self-excisable, full-length RhCMV BAC is demonstrated. Viral progeny with a residual loxP site within the genome were efficiently reconstituted by transfecting pRhCMV/BAC-Cre into rhesus fibroblasts, and reconstituted virions retained the wild-type phenotype both in vitro and in vivo. By analyzing individual RhCMV BAC clones, we also show that (i) the unique components of the RhCMV genome do not invert during viral replication, (ii) heterogeneity at the S terminus of the RhCMV genome may be attributed to the presence of a variably reiterated 750-bp sequence, and (iii) the terminal heterogeneity results from viral DNA replication and/or packaging.

MATERIALS AND METHODS

Cells, viruses, and plaque assays. Propagation of RhCMV strain 68-1 (ATCC VR-677) (4) and RhCMV-enhanced green fluorescent protein (EGFP) (12) in telomerase-immortalized rhesus fibroblasts (Telo-RF) (20) has been described previously (11). Virus stock preparations and the determination of virus titers by plaque assays on Telo-RF were performed as previously described (11). Viral replication kinetics were determined by single-step growth curve analyses according to previously reported methods (11). In brief, Telo-RF cultured in six-well plates at a density of 5 × 10⁴ cells/well were infected in triplicate at a multiplicity of infection (MOI) of 0.1. Supernatants from infected cultures were collected daily for plaque assays.

Plasmid construction. To construct the BAC vector pWC155 (Fig. 1A), the EGFP expression cassette excised from pWC139 (12) was cloned into the HindIII site of the pBeloBAC11 vector (New England Biolabs) (1), a derivative of pBAC8L (38). For construction of the simian virus 40 (SV40) promoter-driven expression cassette pWC162, the AgeI/NorI fragment of pWC132 (12) was replaced with an AgeI-XhoI NorI oligonucleotide adapter (5'-GGCCGCGAAAT TTTCTGAGA-3' and 5’-CCGGTCTCGAGAAATTTGCG-3’). The SV40 promoter-polyadenylation signal region from pWC162 was PCR amplified with primers PAB507 (5'-AACCCGGGTGACGTTAGGTTGAAAG-3’) and PAB508 (5’-CCTCCCGCGTCGACAACTAGAATGCAGTGAAA-3’), then cloned into the TOPO TA cloning vector (Invitrogen), resulting in pWC165.

For construction of the Cre/lox recombination vector, pWC212, a two-step replacement procedure for allelic exchange was performed (3, 6, 31). Briefly, the delivery vector, pWC212, was electroporated into DH10B (Invitrogen) by electroporation according to published methods (38). To substitute the Cre cassette for the EGFP cassette of RhCMV/BAC-EGFP plasmid, a two-step replacement procedure for allelic exchange was performed (3, 6, 31). Briefly, the delivery vector, pWC212, was electroporated into DH10B containing pRhCMV/BAC-EGFP. Cointegrates of the BAC and pWC212 were selected by cultivation on agar plates containing chloramphenicol (25 μg/ml) and kanamycin (30 μg/ml) at 30°C. Colonies were streaked onto new chloramphenicol plates and incubated at 30°C to allow resolution of the cointegrates. Resolved clones were selected by incubation on chloramphenicol plates containing 5% sucrose at 30°C and subsequently tested for the resulting sensitivity to kanamycin. The recombinant BAC clones were further screened by PCR with the

FIG. 1. Strategy for constructing a self-excisable RhCMV BAC. (A) Cloning of the full-length RhCMV genome into a BAC vector by Cre/lox recombination. The RhCMV genome structure with expansion of the US1 to US3 region of recombinant viruses is diagrammed. The open box represents the internal junction between L and S components of the RhCMV genome. Cre/lox recombination was performed in Telo-RF cells. Recombinant clones containing the BAC vector in the viral genome (vRhCMV/BAC-EGFP) were plaque purified, and circular-form viral DNA was transformed into E. coli strain DH10B for plasmid isolation. (B) Substitution of the cre ORF for the egfp ORF by allelic exchange in E. coli. Only a portion of each plasmid is illustrated. The homologous flanking regions and the cre ORF of the recombinant vector, pWC212, are illustrated. UL, unique sequences of the L component; US, unique sequences of the S component; PSV40, SV40 promoter; poly A, SV40 polyadenylation signal.
primer pairs PAB509-PAB510 and PAB431-PAB489 (12), which are specific for the Cre and EGFP cassettes, respectively.

Plasmid transfection and virus reconstruction. Optimal conditions for transfecting Telo-RF with FuGENE 6 reagent (Roche Applied Science) have been described. Briefly, cells were seeded at a density of 2 × 10^6 cells/well 24 h prior to transfection. Transfection reagent-plasmid DNA mixtures in a ratio of 3 μl/μg were added directly to the cultures. For reconstruction of the virus from the BAC plasmids, transfected cells were subdivided (1:3) 4 to 5 days posttransfection and cultured until plaque development. For diagnostic PCR, supernatants collected from transfected or infected cultures were heated at 80 °C for 10 min and used as templates. The primer pairs PAB431-PAB510 and PAB509-PAB510 were used to amplify the viral US1-US2 region and Cre ORF, respectively. PCR amplicons were cloned and verified by sequencing.

Viral DNA and BAC plasmid preparation and analysis. RhCMV nucleocapsid DNA was isolated according to the methods developed by Sinzger et al. (39). In brief, infected cells were harvested when cultures reached 100% cytopathic effect (CPE), collected by low-speed centrifugation, and washed twice with cold phosphate-buffered saline. Cells were resuspended in cell permeabilization buffer (10 mM Tris-HCl [pH 7.5] containing 300 mM sucrose, 5 mM MgCl2, and 1% Triton X-100), incubated on ice for 10 min, and then treated with micrococcal nuclease (1,500 U/ml; U.S. Biochemicals) at 37 °C for 60 min. After nuclease treatment, cells were digested with protease K (100 μg/ml; Invitrogen) at 50 °C overnight, and the DNA was extracted with phenol-chloroform and precipitated with isopropanol. BAC plasmid DNA was isolated from E. coli by using an alkaline lysis procedure with the NucleoBond Plasmid Maxi kit (Clontech Laboratories) according to the manufacturer’s instructions. Restriction endonuclease-digested DNA was resolved by electrophoresis on 0.8% agarose gels for 18 h at 40 V and visualized on UV transilluminator.

Construction of the full-length, self-excisable RhCMV BAC. To clone the full-length RhCMV genome as a self-excisable BAC in E. coli, the strategy described by Smith and Enquist for the successful construction of the PRV BAC (41), was applied. This method is based on Cre/lox site-specific recombination to cross the BAC vector into the viral genome (Fig. 1A). The BAC vector was engineered into the 210-bp intergenic region of the RhCMV genome between the US2 polyanalyses signal and the US1 transcriptional start site. Our previous study demonstrated that insertion of the EGFP expression cassette into this region does not alter the pathogenicity of the recombinant virus (12). Telo-RF cultures were transfected with the Cre expression vector pOG231 (32) and infected with RhCMV-EGFP (MOI of 1) 2 days after transfection. Supernatants were collected when the cells exhibited 100% CPE and transferred to fresh Telo-RF cultures in 10-fold serial dilutions. After four rounds of plaque purification, GFP+ clones (RhCMV-loxP) were isolated, corresponding to viral genomes in which the EGFP cassette and one loxP site had been excised (Fig. 1A). The US1-US2 region of the RhCMV-loxP genome was PCR amplified, cloned, and sequenced to confirm the integrity of sequences (Fig. 4B).

To insert the BAC vector pWC155 (Fig. 1A) into the RhCMV genome, Telo-RF were cotransfected with pWC155 and pOG231 and then infected with RhCMV-loxP the following day. Supernatant was collected 48 h postinoculation (hpi) after the completion of one round of RhCMV replication (11) and serially diluted onto fresh cell cultures. Since it has been observed that oversized MCMV genomes are not stable in cell cultures (M. Messere, unpublished data), only two rounds of GFP+ plaque purification were performed. This was done to reduce the possibility of selecting RhCMV recombinants with deletion of nonessential gene loci. Viral DNA was purified from the infected cells after circularization of the linear viral genome and transferred into E. coli. Large-molecular-weight plasmids exhibiting restriction endonuclease digestion profiles identical to that of wild-type RhCMV (except for changes resulting from insertion of the BAC vector) were isolated from the chloramphenicol-resistant clones (Fig. 2B, lane 1). Transfection of these BAC clones into rhesus fibroblasts led to the development of GFP+ plaques and the reconstitution of RhCMV/BAC-EGFP.

The design of the self-excisable BAC is to constitutively provide Cre recombinase activity to maximize intragenomic recombination at two loxP sites until the BAC vector is removed from the viral genome. The best location to insert the Cre expression cassette is within the BAC vector so that only the virus carrying the vector in its genome expresses the recombinase. To replace the egfp ORF within the SV40-driven expression cassette with the cre ORF, allelic exchange was performed by using a two-step replacement procedure in E. coli, resulting in pRCMV/BAC-Cre (Fig. 1B). The recom-
Combined clones were screened by using PCR with primer sets specific to either ORF to confirm allelic exchange occurred at the correct region of the plasmid (data not shown).

**Characterization of the RhCMV BAC plasmids.** RhCMV BAC plasmids were digested with multiple restriction enzymes and compared to the wild-type nucleocapsid DNA after electrophoresis. Both pRhCMV/BAC-EGFP and pRhCMV/BAC-Cre exhibited wild-type restriction profiles, except for the novel fragments generated by insertion of the BAC vector into the US1-US2 region (Fig. 2A). Both plasmids contained additional restriction fragments of correct molecular sizes after digestion with multiple restriction endonucleases (Fig. 2B, lanes 1 and 2). Consistently, the 9.3-kb SalI and the 25.7-kb EcoRI wild-type fragments, encompassing the site of recombination, were only observed with wild-type nucleocapsid DNA (Fig. 2B, lanes wt). Comparison of the restriction patterns of the BAC-containing variants with wild-type RhCMV DNA indicated that there were no overt changes in the fidelity of the viral genome during propagation and allelic exchange in bacteria or during infection in mammalian cells. Due to the circular nature of BAC plasmids, novel restriction fragments resulting from the fusion of the termini in the BAC plasmids are marked with the joined symbols. Size standards indicated in kilobases are displayed to the left of the gel.

![Fig. 2. Characterization of RhCMV BAC plasmids.](image-url)
of plasmids (C1, C2, and C3) were observed in the isolated pRhCMV/BAC-EGFP clones, each defined by a unique EcoRI fragment not found in the other two BAC clones (Fig. 3A). The C1 plasmid contained a unique 2.8-kb fragment, whereas 3.6- and 4.4-kb fragments were observed in the C2 and C3 plasmids, respectively. The difference in sizes between the unique fragments of C1 and C2 and of C2 and C3 were both ~750 to 800 bp. None of these fragments were found in the linear genomic DNA of wild-type RhCMV (Fig. 2B) or the BAC-reconstituted virus, RhCMV/BAC-EGFP (data not shown). It is unlikely that these fragments resulted from the random deletion of viral sequences because the BamHI digestion profiles of these plasmids also presented unique fragments with the same size difference (data not shown). Since the BAC DNA is maintained in a covalently closed circular form during propagation in *E. coli*, restriction enzyme digestion would generate a fragment that spanned the L and S termini, which would not be present in the linear-form viral DNA packaged in mature virions. To confirm the nature of these heterogeneous fragments, the fused termini of the viral genome were ampli-
fied from the RhCMV BAC by PCR, cloned, and used for the preparation of hybridization probes (Fig. 3B). The pac1 motif (GGGGGGGTGTGTTGCGGCGG) was detected in all the probes from the RhCMV BAC clones. Interestingly, there is no pac2-like sequence located at the termini of the RhCMV genome. The distance from the pac1 motif to the cleavage site for herpesviruses is uniformly 30 to 35 bp (summarized in reference 26). Accordingly, the predicted sizes of the terminal EcoRI fragments for the RhCMV genome are 2.32 and 0.52 kb (L and S, respectively), and the sum of these two fragments is consistent with size of the smallest unique EcoRI fragment from Cl (Fig. 3A). Radiolabeled probes LS, L, and S all hybridized to the unique EcoRI fragments of the three BAC plasmids (Fig. 3C, only probe L is shown), indicating that these fragments were derived from the fusion of L and S termini of the RhCMV genome.

The differences in the sizes of the fused L-S fragments within the different BAC clones suggested that there was heterogeneity in the composition of the termini of the RhCMV genome. To test this possibility, hybridization analysis of linear virion DNA was performed with L, S, and LS probes. All three probes hybridized with multiple bands, most of which were commonly detected by every probe (Fig. 3D). Each commonly detected band (Fig. 3D, labeled as fragments B to E) was detected by every probe (Fig. 3D). The presence of multiple hybridizing bands in DNA purified from virions suggested either of two possibilities. The RhCMV 68-1 virus stock may have consisted of a heterogeneous population, with each subpopulation characterized by a variably sized S terminus. Alternatively, S-terminal heterogeneity may have resulted as a consequence of viral replication. To test the source of variation in the length of the S terminus, fibroblasts were transfected with the C3 form of pRhCMV/BAC-EGFP. Circular-form viral nucleocapsid DNA was prepared from the progeny infected cells and used to transform E. coli. Among the 12 progeny BAC isolates, 8 exhibited the progenitor C2 conformation. The remainder of the clones exhibited the C3 restriction profile (Fig. 3E, only five clones are shown). The fact that different genomic forms were recovered after transfection with the C3 BAC indicates that the heterogeneity at the S terminus of the RhCMV genome resulted from viral DNA replication and cleavage or packaging.

RhCMV exhibits simple genome structure. Herpesviruses with complex genome features, such as class D (e.g., varicella-zoster virus) or class E (e.g., HSV and HCMV) genomes, contain two or four isomeric forms in the virions, respectively. Since the genome feature of RhCMV has not been fully elucidated yet, we utilized various BAC clones to examine whether the RhCMV genome isomericizes. The L and S components of viral genomes do not invert during propagation in recombination-deficient E. coli (7, 23); therefore, each BAC clone represents the fixation of an individual genomic isomer. Due to the circular nature of plasmids, either class D or class E genomes should be present as two different conformations in the BACs. Restriction analyses of various isolated RhCMV BAC clones demonstrated that different isoforms of RhCMV genome do not exist in mature virions. The 8.2-kb EcoRI fragment flanking the internal L-S junction (Fig. 2A) is present in all of the examined pRhCMV/BAC-EGFP isolates (Fig. 3A and E). It has been demonstrated that the inversion of L and S components of HSV and CMV is mediated by repeated sequences (or a sequences) located at both the genomic termini and the L-S junction (13, 19). In addition, two conserved sequence motifs within the a sequences of both viruses, pac1 and pac2, are known to be important in the packaging and cleavage of viral genomes during replication. In RhCMV, a pac1-like motif was identified within the internal L-S junction by sequence alignment (GGGGGGGTGTGTTGCGGCCGGGGGGG, GenBank accession no. AF474179). This sequence is not a duplication of the pac1 motif located within the L terminus, as in the case of HCMV. Further, the restriction fragments containing this internal pac1-like sequence did not hybridize with the probes specific for L or S termini (data not shown). The absence of a positive hybridization signal revealed that there is a lack of an a-sequence-like repeat element in the RhCMV genome.
Efficient reconstitution of vector-free viruses from the BAC. pRhCMV/BAC-Cre was transfected into rhesus fibroblasts to reconstitute vector-free RhCMV progeny. Visible plaques developed between 7 to 10 days posttransfection from five independent transfections. To evaluate the efficiency of the BAC vector elimination from the viral genome after delivery into eukaryotic cells, progeny virions in the supernatant were collected and examined by PCR. Remarkably, the BAC vector was excised from the viral genome very quickly (possibly before the first round of viral replication was completed). The ~1.0-kb wild-type-like fragment was amplified with primers specific to US1 and US2, whereas cre was undetectable by diagnostic PCR (data not shown), indicating the complete excision of the BAC vector from the viral genome. The BAC-reconstituted virus, RhCMV-loxP(r), was passaged on fresh Telo-RF cultures twice, and the supernatant was collected for virus stock preparation when 100% of the cells exhibited CPE. Nucleocapsid DNA was isolated from infected cells and digested with multiple enzymes. The restriction patterns of the RhCMV-loxP(r) genome were identical to those of its parental strains, wild-type RhCMV and RhCMV-loxP (Fig. 4A, only EcoRI patterns are shown).

Sequence integrity and gene expression profiles of the BAC-reconstituted virus. To investigate the integrity of viral sequences at the region where multiple rounds of recombination occurred, the diagnostic PCR product for US1-US2 region of RhCMV-loxP(r) was cloned and sequenced. The sequence of the BAC-reconstituted virus within this region was identical to the corresponding sequence of wild-type RhCMV, except for the residual 34-bp loxP site that was retained following excision of the BAC vector (Fig. 4B). To assess whether the residual loxP sequence would affect the expression of the neighboring ORFs (US1 to US3), the steady-state levels of these transcripts in infected cells were analyzed by 3' RACE. The temporal expression profiles of US1, US2, and US3 of RhCMV-loxP(r) were indistinguishable from those of wild-type RhCMV (Fig. 4C). Interestingly, even with the ~9-kb BAC vector inserted upstream of the US1 ORF, US1 transcription of RhCMV/BAC-EGFP remained comparable to that of the wild-type virus (data not shown), suggesting that this region can tolerate large exogenous DNA sequences without disrupting the expression of surrounding ORFs.

BAC-reconstituted RhCMV retains wild-type replication properties. To characterize the replication properties of reconstituted virus in vitro, a single-step growth curve analysis was performed in Telo-RF cultures. The presence of the BAC vector in the RhCMV genome partially inhibited viral replication in cell cultures (Fig. 5A). RhCMV/BAC-EGFP replicated at a slower rate, and the progeny viral yields were reduced 10-fold at later stages of infection. This compromised growth phenotype was similar to that of HCMV carrying the BAC vector in its full-length genome (52). In contrast, RhCMV-loxP(r) exhibited replication kinetics and viral yields similar to those of both wild-type RhCMV and RhCMV-loxP (Fig. 5A).

Although the genome structure and in vitro replication properties of RhCMV-loxP(r) were indistinguishable from those of the wild-type virus, some alterations in the viral genome may have occurred during propagations in E. coli. Therefore, the infectivity of RhCMV-loxP(r) in rhesus monkeys was examined to assure that this BAC plasmid could be used as the parental clone to construct mutants for future in vivo studies. Two healthy, seronegative rhesus monkeys were intravenously inoculated with 1.8 × 10^7 PFU of RhCMV-loxP(r). Viral DNA loads in plasma were longitudinally evaluated by a real-time PCR assay, and RhCMV-specific antibody titers were measured by enzyme-linked immunosorbent assay. RhCMV-loxP(r) exhibited replication kinetics in macaques similar to those observed in previous studies with the wild-type RhCMV strain 68-1 (37). RhCMV DNA was first detected in the plasma samples on 3 to 5 days postinoculation (dpi) with peak copy numbers observed on 7 dpi (Fig. 5B). RhCMV DNA copy numbers subsequently declined to undetectable levels by 21 dpi. Both animals developed specific anti-RhCMV humoral immune responses between 2 to 3 weeks postinoculation (immunoglobulin G titer, >1:50), and the antibody titers maintained at high levels even though RhCMV DNA was no longer detectable in the plasma samples (Table 1). The endpoint anti-RhCMV antibody titers were comparable to those for occludin.
macaques naturally infected with RhCMV (data not shown). Consistent with the establishment of persistent RhCMV infection, IE1-positive cells and viral DNA within the spleen tissues of MMU29836 (terminated at 27 weeks postinoculation) were detected by immunohistochemistry and PCR, respectively. The replication kinetics of these viruses were compared to those of wild-type RhCMV and RhCMV-\textit{loxP}. Telo-RF cells were infected in triplicate with each virus at an MOI of 0.1. Datum points represent the mean of infectious virus titers in the supernatants of three independent cultures with the standard deviations indicated by the error bars. (B) Longitudinal viral loads in the plasma of two seronegative rhesus monkeys intravenously inoculated with RhCMV-\textit{loxP} (r). Plasma samples were collected and processed for DNA isolation at the time points as indicated. RhCMV DNA copy numbers were quantified by a real-time PCR assay with the detection limit of 200 copies. Datum points represent the averages of two independent real-time PCR analyses.

**DISCUSSION**

The strategy for cloning the full-length RhCMV genome into the BAC vector was based on (i) the broad applicability of Cre/\textit{lox} system to herpesvirus genomics, which has been demonstrated in the construction of PRV and HCMV BACs (41, 52), and (ii) the fact that US1-US2 intergenic region of RhCMV is amenable to insertion of foreign DNA without losing the wild-type phenotype (12). The self-excising RhCMV BAC was constructed after two rounds of Cre/\textit{lox} recombination performed in mammalian cells and an allelic exchange constructed in \textit{E. coli}. Upon delivery of the RhCMV/BAC-Cre plasmid into rhesus fibroblasts, the vector was autonomously removed from the viral DNA, with only one residual \textit{loxP} site retained in the genome of reconstituted progeny. The presence of either the 34-bp \textit{loxP} sequence or the 9-kb BAC vector did not disrupt the expression profiles of neighboring genes (US1 to US3) of the insertion site. Further, virus reconstituted from pRhCMV/BAC-Cre replicated with wild-type kinetics in cell culture, and the temporal detection of viral DNA in plasma samples, as well as the persistent expression of viral antigens, after intravenous inoculation in seronegative macaques was similar to that seen with the parental strain RhCMV 68-1 (37).

Insertion of the BAC vector into the CMV genomes has typically required the deletion of viral sequences that are non-essential for in vitro replication because CMVs only tolerate up to ∼5 kb of additional DNA in their genomes (7, 24, 27). Progeny viruses reconstituted from these BACs are not genetically wild type. Therefore, restoration of the deleted viral sequences to the BACs by using the mutagenesis tools in \textit{E. coli} is necessary (18, 49). For genetic analyses of RhCMV pathogenesis, the complete RhCMV genome should be maintained in the clone to reduce the possible attenuation of reconstituted progeny. The adaptation of the Cre/\textit{lox} approach in the present study reemphasized that a full-length CMV genome can be directly cloned into the BAC vector without the need to delete or disrupt viral ORFs. In addition, the self-excising system minimizes the risk of generating spontaneous deletions (40; M. Messerle, unpublished) or attenuation (2, 49) arising from the oversized viral genome. The latter consideration reflects a limitation of the amount of DNA that can be encapsidated into mature virions. Inclusion of up to 9 kb of BAC DNA in other non-CMV herpesvirus BAC constructs has not been reported to interfere with the kinetics of viral replication in vitro (2, 23, 40, 53). In contrast, both HCMV (52) and RhCMV that harbor the BAC vector in the viral genome exhibit compromised replication kinetics in cell culture. One interpretation of these observations is that CMV may have a more stringent size constraint for packaging than other herpesviruses. Consistent with this interpretation is the fact that the expression patterns for US1 to US3 of RhCMV/BAC-EGFP

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<sup>a</sup> Titers are presented as the inverse of the last dilution of plasma with an optical density value greater than 0.1 absorbance units above the average negative control optical density value.

<sup>b</sup> The starting titer for plasma dilutions was 1:12.5.

<sup>c</sup> ND, not done (samples not available).

<sup>d</sup> The animal was euthanized for reasons unrelated to RhCMV infection.
were unchanged from the wild-type patterns. Our results suggest that the altered replicative phenotype may be attributed to the oversized genomes that exceeded the limitation of the packaging capacity, not to interference with viral gene expression.

During the course of the present study, it was noted that there were three discrete size conformations of the RhCMV genome cloned into the BAC vector. These clones, C1 to C3, were indistinguishable from each other after endonuclease digestion, except for a single EcoRI (or BamHI) restriction fragment specific to each clone. Hybridization analysis of virion DNA demonstrated that the terminal region of the RhCMV S component is variably reiterated (containing one of the fragments A to E). The vast majority of virions contained either fragments B or C at the S terminus. A comparison of hybridizing fragments of plasmids C1 to C3 with virion DNA indicated that the BAC clones contained only a single fragment that corresponded in size to the end-to-end fusion of the L terminus and one of these S fragments. Many herpesviruses, including HCMV, contain terminal and/or internal reiterated sequences (33). Due to the variation in the number of the reiterations in the viral genome, the size of the individual encapsidated genome can vary. Heterogeneity has been observed at the genome termini and the L-S junction of HSV-1 (34) and multiple strains of HCMV, including the AD169, Towne, and Davis strains (16, 28, 42, 43, 46, 47). Within the HSV-1 genome, one copy of the a sequence is located at the S terminus, and one to several copies are located both at the L terminus and the internal L-S junction (34). The terminal genome structures of various HCMV strains are less consistent, but in general have one or no copy of the a sequence at the S termini and one to several copies at the L termini. Guinea pig CMV, with a simple genome structure and a simple terminal repeat arrangement, contains either one copy of a 1-kb direct repeat sequence at each end (type II genome) or one repeat at left end and no repeat at the other (type I genome) (25).

The hybridization results suggest that the mature RhCMV genomes contain various numbers of repeat sequences at the S terminus. The ladder patterns of hybridizing bands detected with the L probe, each differing in size from the preceding band by 750 bp, cannot be reconciled with the restriction map of the L terminus. Only the hybridizing 2.32-kb EcoRI and 1.38-kb BamHI fragments (Fig. 3D, marked with asterisks), accounting for the L-terminal fragments of the RhCMV genome, were observed. Infectious virions with S-terminal heterogeneity were reconstituted from the C1 prRhCMV/BAC-EGFP (possessing the smallest EcoRI fragment of 2.8 kb), indicating that C1 plasmid contained the elements essential to generate the 750-bp repeat. The same terminal ladder patterns (both EcoRI and BamHI) were detected by using probes specific to the L or S termini (Fig. 3D, fragments B to E). Commonality of hybridizing bands with probes derived from opposite ends of the genome constitutes prima facie evidence that these fragments are comprised of both L and S terminal sequences. These findings raised two possible explanations for generation of the 750-bp reiterated sequence. Since the size of this repeat was larger than the EcoRI fragment of the S terminus (~520 bp) at the S terminus of the corresponding linear-form viral DNA, one possible model is that the repeat structure is a "hybrid" containing sequences from both L and S termini of the RhCMV genome. However, this would require complicated processes of recombination or ligation during the viral DNA cleavage or packaging to generate. The more likely model is that the repeat, which may be derived from a process involving self-amplification during the replication or cleavage or packaging of viral genome such as HSV (14), consists of sequences derived entirely from the L terminus with no sequences from the S terminus (Fig. 3F). According to this model, the S terminus of the linear RhCMV genome contains from zero (type A) to at least four (type E) copies of the 750-bp reiterated sequence (Fig. 3F, only types A to C are illustrated). The encapsidated unit-length molecules may be generated by cleavage between the adjacent copies of this repeat within the replicative intermediates (Fig. 3F), similar to the cleavage of adjacent a sequences in the concatemeric genome of HSV (29). Another scenario is that the S-terminal heterogeneity of RhCMV genome is derived from the duplicative and nonduplicative cleavage process, similar to what has been shown in guinea pig CMV (30). However, our model does not explain the reason that heterogeneity only occurs at the S terminus of the viral genome. It is possible that the cleavage of concatemer always occurs near the first pac1 motif from the L terminus. Or, another trimming mechanism for the L terminus during or after genome cleavage or packaging is responsible for its sequence consistency. The nature and precise structure of these heterogeneous fragments at the S terminus of RhCMV genome are currently under investigation. Similar to other herpesviruses, RhCMV also contains variable numbers of a terminal element at the termini of its genome. There are at least five types of RhCMV genome that can be identified from the virion DNA. According to the results obtained from restriction and hybridization analyses, the predominant forms of RhCMV genome are type B and type C. The intensities of hybridization to bands B and C are consistent with the observation that C2 and C3 BAC comprised all of the 12 progeny BAC isolates derived after transfection with C3 plasmid. These findings support the hypothesis that the heterogeneity of the RhCMV S terminus results from viral DNA replication or packaging, and the three original BAC clones faithfully reflect these isomers.

In conclusion, the construction of a full-length, self-excisable BAC clone amenable to genetic analysis of RhCMV is described here. Virions reconstituted from this self-excisable BAC after transfection into fibroblasts were vector-free and differed from wild-type RhCMV merely by the 34-bp residual loxP sequence within the viral genome. This exogenous sequence has no effect on either the expression of neighboring genes or the in vitro and in vivo replication properties. With the results presented here, there are now three BAC-cloned, full-length animal herpesviruses—MCMV (49), murine gammaherpesvirus 68 (1), and RhCMV—that are known to retain wild-type replication properties in their respective animal hosts after the excision of vector sequences. It is especially noteworthy that the BAC system is applicable for the studies of herpesviral pathogenesis in mouse and nonhuman primate models. In the case of the slowly replicating betaherpesviruses, such as HCMV and RhCMV, applying the BAC technology and the tools for mutagenesis in E. coli is the preferred method for genetic manipulation. The Cre-mediated excision of the BAC vector from the viral genome can be completed after only one
round of viral replication in permissive cells, which will greatly facilitate genetic analyses of RhCMV. This RhCMV BAC, pRhCMV/BAC-Cre, will serve as the basis for construction of RhCMV mutants to study CMV pathogenesis in the rhesus macaque model.

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