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Analysis of Human Cytomegalovirus *ori*Lyt Sequence Requirements in the Context of the Viral Genome

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During the lytic phase of infection, replication of herpesvirus genomes initiates at the lytic origin of replication, *ori*Lyt. Many herpesviruses harbor more than one lytic origin, but so far, only one *ori*Lyt has been identified for human cytomegalovirus (HCMV). Evidence for the existence of additional lytic origins of HCMV has remained elusive. On the basis of transient replication assays with cloned viral fragments, HCMV *ori*Lyt was described as a core region of 1.5 kbp (minimal *ori*Lyt) flanked by auxiliary sequences required for maximal replication activity (complete *ori*Lyt). It remained unclear whether minimal *ori*Lyt alone can drive the replication of HCMV in the absence of its accessory regions. To investigate the sequence requirements of *ori*Lyt in the context of the viral genome, mutant genomes were constructed lacking either minimal or complete *ori*Lyt. These genomes were not infectious, suggesting that HCMV contains only one lytic origin of replication. Either minimal or complete *ori*Lyt was then ectopically reinserted into the *ori*Lyt-depleted genomes. Only the mutant genomes carrying complete *ori*Lyt led to infectious progeny. Remarkably, inversion of the 1.5-kbp core origin relative to its flanking regions resulted in a replication-defective genome. Mutant genomes carrying minimal *ori*Lyt plus the left flanking region gave rise to minifoci, but genomes harboring minimal *ori*Lyt together with the right flanking region were noninfectious. We conclude that the previously defined minimal lytic origin is not sufficient to drive replication of the HCMV genome. Rather, our results underline the importance of the accessory regions and their correct arrangement for the function of HCMV *ori*Lyt.

Human cytomegalovirus (HCMV), like all herpesviruses, has at least two different modes of infection, either lytic replication or latency. Whereas the replication mode of the HCMV genome during latency is rather elusive, substantial progress has been made in dissecting the molecular steps that occur during lytic DNA replication (35, 39; for a review, see reference 41). Following cell entry, HCMV capsids are transported to the nuclear pores, where they release the linear genomes into the cell nucleus. Then, the linear genomes are rapidly circularized and serve as templates for transcription and replication. It has been proposed that the initial steps in DNA replication of herpesviruses take place by a theta-like mechanism, followed by a rolling-circle mechanism of replication at later stages of the lytic infection cycle (reviewed in references 36 and 46). This view of herpesvirus DNA replication has recently been challenged by the observation that genome circularization seems not to be a prerequisite for replication of herpes simplex virus (HSV) and the suggestion that linear genomes serve as replication templates (31). It remains to be shown whether the same applies to HCMV and other herpesviruses as well.

Regardless of which mechanism is used, lytic replication of herpesviruses initiates at defined regions of the viral genomes, the so-called lytic origins of replication (*ori*Lyt). One *ori*Lyt has been defined for HCMV (1, 2, 27, 37). By transient-replication assays with cloned subgenomic fragments, *ori*Lyt was located upstream of the gene encoding the single-stranded DNA binding protein (UL57) approximately in the middle of the unique long (UL) segment (1, 37). In contrast to the origins of alphaherpesviruses, HCMV *ori*Lyt is remarkable for its apparent size and complexity, and it seems to lack any obvious sequence and evolutionary homology to other viral DNA replication origins. Despite the missing sequence homology, however, there is a striking resemblance in the structural organization among the origins of human, chimpanzee, rhesus, rat, and mouse CMVs, e.g., an asymmetric base distribution with an A+T-rich region located at one end and an extended G+C-rich segment at the other end (20, 28, 38, 53). HCMV *ori*Lyt contains numerous reiterated and inverted sequence elements, including known consensus transcription factor recognition sites. Although the analysis of these sequence elements in either *ori*Lyt function or local transcription regulation has just begun (33), their distribution resembles the organization of some complex transcriptional enhancers, and the existence of these binding sites suggests the presence of putative promoter regions. Transcriptional control elements and enhancers are indeed common components of many viral and eukaryotic origins of DNA replication (21, 42, 48, 50, 51). Given the complex structure of HCMV *ori*Lyt, the lack of an apparent *trans*-acting factor comparable to the HSV origin-binding protein (41), and the recent identification of intraorigin RNA transcripts that in part form stable and persistent RNA-DNA hybrid structures within *ori*Lyt (45), the mechanism of replication initiation in HCMV may be different from that employed by other herpesviruses.

Several efforts have been undertaken to define the sequence requirements and boundaries of the HCMV replicator (1, 2, 37, 59). Using transient replication assays, Masse et al. mapped a 1.5-kbp core region of *ori*Lyt (i.e., minimal *ori*Lyt) to a region

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situated between nucleotides (nt) 92210 and 93715 of the HCMV genome but stated that full replicator activity requires additional sequences of ~1,100 bp to the left and 700 bp to the right of the origin core (37). The complete lytic origin of HCMV was thus determined to be a segment with a size of ~3.3 kbp. Insertion and deletion studies combined with transient replication assays performed by Anders and colleagues and Zhu et al. described a core region of HCMV *ori*Lyt that is similar to that defined by Masse et al. but somewhat shifted to the left, namely, between nt 91751 and 93299 (1, 59). Since the introduction of mutations into the accessory sequences flanking either side of this core had little or no effect on replication efficiency (59), the role of these sequences flanking *ori*Lyt requires further investigation.

Some herpesviruses, including HSV and Epstein-Barr virus, harbor more than one lytic origin of replication (26, 46), whereas to date only one lytic origin has been described for HCMV. However, the existence of other lytic origins in HCMV has not been excluded. Although the significance of multiple origins remains to be determined, this raises the question of whether the HCMV genome contains an additional lytic origin(s).

In order to define the sequence requirements for a functional *ori*Lyt of HCMV, we decided to analyze the significance of the core origin and its flanking regions in the context of the HCMV genome. Also, we asked whether the HCMV genome contains additional lytic origins. Using a bacterial artificial chromosome (BAC)-cloned HCMV genome (6), we constructed a series of mutant HCMV genomes in which we first deleted either the minimal or the complete lytic origin and then, in subsequent rounds of mutagenesis, reinserted minimal or complete *ori*Lyt at an ectopic position of the genomes with the origin deleted. Our results imply that the genome of HCMV strain AD169 contains only one lytic origin. We also provide evidence that the accessory regions flanking the origin core are essential for initiation of DNA replication at HCMV *ori*Lyt. Interestingly, the left flanking region seems to be of greater importance for HCMV *ori*Lyt function than the sequences to the right, since upon transfection, mutant genomes with a fragment encompassing minimal *ori*Lyt plus sequences to the left led to the formation of small foci of green fluorescent protein (GFP)-expressing cells, whereas the sequences to the right plus minimal *ori*Lyt did not.

MATERIALS AND METHODS

Virus and cells. The parental HCMV strain used in this report is the BAC-derived recombinant virus HCMV-GFP (8) originating from the BAC-cloned HCMV AD169 genome (6). Culture of human foreskin fibroblasts (HFF), preparation of virus stocks, growth curves, and determination of virus titers were essentially done as described previously (6). The adenovirus 5 recombinant dl1014—defective for most of the E4 region (11)—was propagated on the E4-complementing cell line W162 (56) (both kindly provided by Gary Ketner, Johns Hopkins Bloomberg School of Public Health, Baltimore, Md.). W162 cells were cultured in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin sulfate per ml. For preparation of replication-deficient, UV-inactivated adenovirus particles, dl1014 was isolated from infected W162 cells by three cycles of freeze-thawing, purified by two rounds of CsCl gradient centrifugation, and inactivated by treatment with psoralen and UV irradiation as described previously (17).

Plasmids. The nucleotide positions of the AD169 genome provided here refer to the GenBank entry with accession number X17403 (13). For construction of pOri6K-Kan1 and pOri6K-Kan4, which contain a kanamycin resistance marker plus one FLP recognition target (FRT) site in different orientations, and the bacterial origin of replication R6Kγ (34), a 1.0-kbp fragment encompassing the kanamycin resistance gene from transposon Tn*903* plus one wild-type FRT site was amplified by PCR using the primers Kanori6k.for (5′-CGC AGA TCT GGT GAC CAC GTC GTG GAA TGC CTT-3′) and Kanori6k.rev (5′-CCC AGA TCT CGA TTT ATT CAA CAA AGC CAC GTT-3′) with pSLFRTKn (3) as a template. The resulting PCR product was digested with BglII and ligated to a 0.5-kbp fragment comprising the R6Kγ origin isolated from plasmid pGP704 (40) (kindly provided by Holger Rüssmann, University of Munich, Munich, Germany) by BamHI digestion. To generate plasmid pOri6K-F5, which harbors the kanamycin resistance cassette flanked by mutant FRT sites, PCR was performed with pSLFRTKn as a template and primers FRT-F5.for (5′-CGC AGA TCT GAA GTT CCT ATA CCT TTT GAA GAA TAG GAA CTT CGC CAG TGT TAC AAC CAA TTA ACC-3′) and FRT-F5.rev (5′-CGA GAA TTC AGA AGT TCC TAT TCT TCA AAA GGT ATA GGA ACT TCC GAT TTA TTC AAC AAA GCC ACG T-3′), which provided mutant FRT sites (49). The PCR product was digested with EcoRI and BglII and cloned into the EcoRI and BglII sites of pGP704. For ectopic insertion of minimal *ori*Lyt, plasmids p6K-ori1.5-3 and p6K-ori1.5-5 were constructed. A 1.5-kbp EcoRI-SacI fragment comprising minimal *ori*Lyt (nt 92210 to 93715 of the AD169 genome) (13) was obtained from pBC-*ori*Lyt (9), which carries the HCMV origin region from nt 92210 to 94860 derived from pCM1029 (25). The fragment was treated with the Klenow fragment of *Escherichia coli* DNA polymerase I and cloned into pOri6K-Kan1 and pOri6K-Kan4, which were cut with EcoRV. The sequence of the 1.5-kbp fragment was determined and compared to the AD169 DNA sequence. We did not find any alterations, except in a region located upstream of the vRNA-2 start site, which was previously reported to exhibit a heterogeneous restriction pattern (45). The sequences equivalent to AD169 nt 93092 to 93218 were missing, and the sequences equivalent to nt 93219 to 93299 were repeated two times. Since the minimal *ori*Lyt is derived from plasmid pBC-*ori*Lyt, which was shown to replicate efficiently in transient replication assays (9), the differences apparently reflect a naturally occurring polymorphism of HCMV *ori*Lyt sequences.

Plasmids p6K-ori3.7-1 and p6K-ori3.7-4, which carry the complete lytic origin, were generated by releasing a 3.7-kbp fragment (nt 91165 to 94860) from HCMV cosmid clone pCM1029 (25) by digestion with DraI and KpnI, followed by insertion into pOri6K-Kan1 and pOri6K-Kan4, which were cut with KpnI and EcoRV. p6K-ori-right, containing the 1.5-kbp origin core plus the right flanking sequences, was constructed by inserting a 2.6-kbp EcoRI-KpnI-fragment (nt 92210 to 94860) derived from pBC-*ori*Lyt into the EcoRV and KpnI restriction sites of pOri6K-Kan-4 after blunting the EcoRI ends using Klenow fragment, and p6K-ori-left originated from pOri6K-Kan-1, which was cut with EcoRV and SacI, followed by insertion of a 2.5-kbp DraI-SacI-fragment (nt 91165 to 93715) obtained from pCM1029. All plasmids containing the R6Kγ origin were propagated in the *E. coli* strain PIR1 (Invitrogen), which provides the phage lambda π protein.

For construction of the shuttle plasmid pST-ori-invers, the oligonucleotides ori-oligo.for (5′-CAG CTG CAC GAT ATC GTG CA-3′) and ori-oligo.rev (5′-CGA TAT CGT GCA GCT GTG CA-3′) were annealed and cloned into the PstI site of pACYC177 (New England Biolabs). The resulting plasmid, pACYC-O, was digested with EcoRV and PvuII, and a 6.1-kbp PvuII-EcoRV fragment (nt 89795 to 95967) isolated from pCM1029 was inserted, leading to pACYC-ori. This plasmid was cut with EcoRI and SacI, releasing DNA sequences (nt 92210 to 94636) that contain the minimal lytic origin, and a 920-bp EcoRI-SacI fragment (encompassing nt 93717 to 94636) amplified by PCR using the primers ori-sac.for (5′-CGC GAA TTC GTT AAC GGG AGC GGG GTC GAC CGC GA-3′) and ori-sac.rev (5′-CCG GAG CTC AGA ACG GCA TGA-3′) was reinserted, creating pACYC-ori-PCR. An HpaI cloning site introduced together with the PCR fragment and located directly adjacent to the EcoRI site was used to insert the minimal *ori*Lyt sequences in inverse orientation. The EcoRI-SalI fragment (nt 92210 to 93727) carrying the minimal *ori*Lyt was obtained from pBC-*ori*Lyt (9) and treated with Klenow polymerase. Finally, the complete insert carrying the inverted minimal lytic origin plus the flanking sequences was isolated as an EcoRV-PvuII fragment and cloned into the SmaI site of shuttle plasmid pST76-KSR (10), resulting in pST-ori-invers.

BAC mutagenesis. Mutant HCMV BACs pHG-1, pHG-Δ1.5, and pHG-Δ3.7 were constructed in *E. coli* DH10B by the ET mutagenesis method (58), which is based on homologous recombination between a linear fragment and the HCMV BAC. The linear fragment encodes antibiotic resistance and is generated by PCR using primers with a size of ~85 bp; 60 nt at the 5′ end of the primers was homologous to the intended integration site in the BAC-cloned CMV genome, and 25 nt at the 3′ end was specific for the antibiotic resistance gene. The recombination functions were provided by plasmid pKD46, which carries the

phage lambda recombination genes red α, β, and γ under the control of an inducible promoter (19). For construction of pHG-1, a PCR fragment was used that contained a tetracycline resistance marker flanked by wild-type FRT sites and that was generated using pCP16 (16) as a template and primers UL1-10ko.for (5′-CAT GGG CAT GCA ATG CAA CAC TAA ATT GTT ATT GCC AGT CGC ACT AAT ACC GGT TGT AAT CAC CAG GGT TTT CCC AGT CAC GAC GT-3′) and UL1-10ko.rev (5′-TCT CAG GTT CCG TAG GGA CTG TCC CTA TAT TCA AAC AGT GGG TTT GAA ACC GGA GAT ATC CAC AGG AAA CAG CTA TGA CCA TGA-3′). To generate mutant BACs pHG-Δ1.5 and pHG-Δ3.7, a PCR fragment encoding kanamycin resistance was used, which was obtained by PCR with plasmid pOri6K-F5 and primers ori15ko.for (5′-TGA CAA GCC TAC GTC ACA CTC GCG TGA CCA CAC CCA CTC CGG ATA TAC GTC ATC CTG TGG CCC CGA AAA GTG CCA CCT GCA GAT-3′) and ori15ko.rev (5′-GCC GCC GAG AGG GAG CCG CCG CGA GAC CCG GAA GCC GTC GCG GTC GAC CCC GCT CCC GGA GTG ACA CAG GAA CAC TTA ACG GCT GA-3′) and ori37ko.for (5′-CCC CCG ACA CGT ACT CGA AGA CCT TAC AGT TTA CGA GTC AAT AAA ACA GGA AAA GAT CCG ACC CCG AAA AGT GCC ACC TGC AGA T-3′) and ori37ko.rev (5′-GCG CCC TCT AGG TAG CAT CAC GGA ACA GGC GGC GAC CGC GGA CTC GCC TCG GCC AGG GGG GTG ACA CAG GAA CAC TTA ACG GCT GA-3′), respectively. Excision of the FRT-flanked antibiotic resistance markers was achieved through FLP-mediated recombination in *E. coli* as described previously (7, 16). HCMV BAC pHG-10 was constructed by a two-step replacement procedure using the shuttle plasmid pST-ori-invers and HCMV BAC pHG-Δ1.5 essentially as described previously (6). In brief, *E. coli* bacteria containing pHG-Δ1.5 were transformed with the shuttle plasmid and incubated at 30°C on agar plates containing 17 μg of chloramphenicol/ml and 25 μg of kanamycin/ml. Clones harboring cointegrates were selected by streaking the bacteria on agar plates containing chloramphenicol and kanamycin and incubation at 43°C. The bacteria were then restreaked on agar plates containing chloramphenicol only and grown at 37°C overnight. Selection of clones that had resolved the cointegrates was done on agar plates containing chloramphenicol plus 5% sucrose at 30°C. Finally, BAC DNA was isolated from 10-ml cultures and checked for the presence of the inverted sequences by restriction analysis.

Mutant BACs pHG-2 and pHG-3 were generated by FLP recombinase-driven integration of plasmids p6K-ori1.5-3 and p6K-ori1.5-5. For this purpose, bacteria harboring HCMV BAC pHG-Δ3.7 were transformed with plasmid pCP20 (16), which encodes FLP. Bacterial colonies were selected on agar plates containing 17 μg of chloramphenicol/ml and 50 μg of ampicillin/ml at 30°C. The bacteria containing both pCP20 and the HCMV BAC were then transformed with either p6K-ori1.5-3 or p6K-ori1.5-5, and clones harboring recombinant BACs were selected by incubation on agar plates containing 17 μg of chloramphenicol/ml and 30 μg of kanamycin/ml at 43°C. BACs pHG-4 and pHG-5 were generated in the same way, i.e., by integration of p6K-ori1.5-3 and p6K-ori1.5-5 into BAC pHG-Δ1.5, and BAC genomes pHG-6, pHG-7, pHG-8, and pHG-9 were obtained by FLP-mediated insertion of p6K-ori3.7-1, p6K-ori3.7-4, p6K-ori-left, and p6K-ori-right into pHG-Δ3.7. The correct integration of the R6Kγ-based plasmids was verified by PCR analysis and sequencing.

Analysis of virus and BAC DNAs. HCMV BAC DNA was isolated from 10-ml overnight *E. coli* cultures by the alkaline lysis procedure (47) and characterized by restriction analysis followed by agarose gel electrophoresis as described previously (6). Large preparations of HCMV BACs were obtained from 100-ml bacterial cultures using Nucleobond PC 100 columns (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Viral DNA was isolated from virions purified from the supernatant of infected cells as described previously (7), and Southern blot analysis of BAC and virus DNAs was performed as reported previously (6). The DNA fragments used for hybridization were either obtained by PCR (probe 1, equivalent to nt 90549 to 90935 of the HCMV genome) using primers orihyb.for (5′-CGG CTT TGC GTC ACC GCC AAC TAA-3′) and orihyb.rev (5′-GCG ATT GAG GTT GCG AGA CCA GAT-3′) with pCM1029 (25) as a template or excised as a 2.2-kbp MluI fragment (probe 2, equivalent to nt 92018 to 94238 of the HCMV genome) from p6K-ori3.7-1.

Accumulation of viral DNA was examined by slot blot assay. HFF were infected at a multiplicity of infection (MOI) of 0.02 PFU/cell with the respective viruses, and total DNA was isolated on days 1, 3, 5, 7, and 9 postinfection using the QiaAmp DNA Blood Mini kit (QIAGEN, Hilden, Germany). One microgram of each DNA sample was transferred to a nylon membrane using a slot blot apparatus. The membrane was then hybridized to a ^{32}P-labeled DNA probe specific for the UL52 open reading frame (ORF). Finally, specific radioactive signals were quantified using a phosphorimager.

Transfection of HCMV BACs. HCMV BAC DNA was transfected into HFF using the adenovirus-mediated gene delivery protocol developed by Cotten et al. (5, 18). In brief, 10 μl of BAC DNA was diluted in 20 mM HEPES, pH 7.4–150 mM NaCl and condensed with polyethylenimine MW 2000 (Sigma) by incubation for 20 min at room temperature. Particles of inactivated adenovirus dl1014 (2×10^9) were added to the mixture and incubated at room temperature for another 20 min. The transfection complexes were applied to the cells, which were kept in serum-free culture medium containing 30 μg of polymyxin B (Sigma)/ml. After 4 h of incubation in a 37°C incubator with 5% CO_2, the fibroblasts were washed twice with PBS and further cultivated in complete cell culture medium. All transfection experiments were done in triplicate. CMV BAC genomes that appeared to be replication deficient were tested in three independent experiments, and transfected cells were incubated until complete cytopathic effect (CPE) had occurred on the fibroblast monolayers transfected with the control BAC pHG-1.

RESULTS

HCMV genomes with a deletion of either the minimal or the complete lytic origin of replication are not infectious. For some herpesviruses, it has been shown that more than one lytic origin is present in the virus genome. In contrast, only one *ori*Lyt has been identified in HCMV (1, 27, 37). Since HCMV *ori*Lyt was primarily defined on the basis of transient replication assays with cloned viral fragments and construction of recombinant genomes with mutated *ori*Lyt has not been possible, the existence of additional lytic origins in the HCMV genome could not be excluded.

In order to address the question of whether the HCMV genome contains other lytic origins apart from the one described, a mutant genome lacking minimal *ori*Lyt was constructed. The design of the mutant was based on the boundaries of *ori*Lyt as defined by Masse et al. (37). They mapped the core of *ori*Lyt between nucleotide positions 92210 (EcoRI) and 93715 (SacI) of the HCMV genome (Fig. 1). Deleting minimal *ori*Lyt was facilitated by applying the ET mutagenesis technique (58), which was recently adapted for the manipulation of BAC-cloned CMV genomes (7, 55) (for technical details, see Materials and Methods). The HCMV BAC pHG-1, a derivative of the previously described HCMV-GFP BAC (8), served as the backbone for construction of all mutant genomes reported in this study (Fig. 2 provides schematic drawings of all mutant genomes). Deletion of the nonessential ORFs UL1 to UL10 in pHG-1 allowed the subsequent insertion of DNA fragments with sizes of up to 6.3 kbp without generating an overlong HCMV genome (see below). The virus derived from pHG-1 expressed the enhanced green fluorescent protein, thereby facilitating the tracking of infected or transfected cells (Fig. 3C), and it showed growth kinetics comparable to those of its parental virus, HCMV-GFP, as well as those of the original BAC-derived virus, RVHB5 (6) (data not shown).

For deletion of minimal *ori*Lyt, a kanamycin resistance (KanR) gene flanked by mutant FRT sites was inserted into the HCMV BAC pHG-1, thereby replacing minimal *ori*Lyt (Fig. 3B). The resulting BAC pHG-Δ1.5-K was characterized by restriction analysis using BglII (Fig. 3A). Following mutagenesis, a fragment of 8.4 kbp characteristic of the parental BAC pHG-1 was replaced by two new fragments of 4.9 and 3.0 kbp in pHG-Δ1.5-K (Fig. 3A, lane 2). The KanR cassette was subsequently excised with FLP recombinase (data not shown), resulting in pHG-Δ1.5 (Fig. 2, line 2).

Transfection of large BACs into primary fibroblasts is associated with notoriously poor efficiency. In general, this does not prevent the reconstitution of replication-competent viruses from BAC genomes, but it may impair the interpretation of

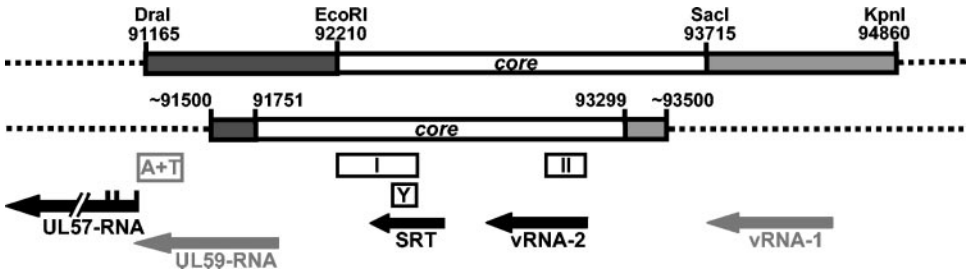

FIG. 1. Schematic of the HCMV *ori*Lyt region. Organization of *ori*Lyt as determined by Masse et al. (upper line) and Zhu et al. (lower line). The origin core is depicted in white, and the left and right auxiliary regions are shown in dark and light grey, respectively. Nucleotide positions are according to Chee et al. Essential elements are shown in black, and elements supposed to be nonessential are in grey. A+T, A/T-rich region; I and II, essential regions I and II; Y, oligopyrimidine sequence. The arrows represent the transcripts detected within and around *ori*Lyt. The 5′ start sites of the UL57 RNA are indicated. Not shown is the 6.5-kb RNA that crosses the origin region. Mutant genomes with a deletion of minimal *ori*Lyt lack the origin core as defined by Masse et al. (nt 92210 to 93715), and mutant genomes with the complete lytic origin deleted lack the sequences between nt 91165 and 94860.

results when replication-incompetent or severely debilitated genomes are analyzed. Therefore, we searched for a more efficient transfection method and tested the adenovirus-mediated gene delivery procedure developed by Baker and Cotten (5, 18). Using this protocol for transfection of HCMV BACs, we obtained an efficiency that was at least 2 orders of magnitude higher than that of any other transfection protocol that we had tested before.

When HCMV BAC pHG-Δ1.5 was transfected into human fibroblasts, single green cells were observed 2 days posttransfection, which remained single for >10 days. No plaques appeared on the cell monolayer (Fig. 3C), and no infectious virus was released into the supernatant. Thus, deletion of minimal *ori*Lyt resulted in a replication-deficient HCMV genome, suggesting that there is no other lytic origin present in the HCMV genome that can substitute for the deleted *ori*Lyt.

The results of a previous study done with viral DNA fragments led to the conclusion that accessory flanking regions of ~1,100 bp to the left and 700 bp to the right of minimal *ori*Lyt are needed to achieve efficient replication originating at HCMV *ori*Lyt (37). In order to evaluate the requirement for these auxiliary regions later, we also constructed a recombinant genome in which the complete *ori*Lyt was deleted. A region of 3.7 kbp extending from nt 91165 (DraI) to 94860 (KpnI) of the HCMV genome was replaced by the KanR marker in mutant BAC pHG-Δ3.7-K (Fig. 3B, line 4). In comparison to the parental BAC pHG-1 (Fig. 3B and A, lane 1), new fragments of 3.9 and 1.9 kbp were present in pHG-Δ3.7-K (Fig. 3A, lane 3). Excision of the kanamycin cassette led to BAC pHG-Δ3.7 (Fig. 2, line 3). Transfection of pHG-Δ3.7 into permissive fibroblasts did not result in the production of infectious progeny (Fig. 3C), providing further evidence that the HCMV genome contains only one lytic origin of replication.

Ectopic insertion of minimal *ori*Lyt does not rescue the replication-deficient phenotype of CMV genomes with the origin deleted. In order to determine the importance of the accessory regions flanking minimal *ori*Lyt, we decided to reinsert either minimal or complete *ori*Lyt at an ectopic position in mutant BACs pHG-Δ1.5 and pHG-Δ3.7, which lack the respective sequences at the original positions (Fig. 2). For insertion of these sequences, we utilized the highly convenient site-specific FLP/FRT recombination system. An FRT site that was inserted at an ectopic position during construction of the parental BAC pHG-1 allowed the insertion of shuttle vectors based on the conditional bacterial R6Kγ replicon (34) and carrying the *ori*Lyt sequences (Fig. 4B) (see Materials and Methods for technical details). Specific targeting of the ectopic locus by the shuttle plasmids was accomplished by using selection markers with wild-type and mutant FRT sites for the deletion of ORFs UL1 to UL10 and of the origin sequences, respectively. Since mutant and wild-type FRT sites cannot interact with each other (49), the shuttle plasmids, which carry a wild-type FRT site, become integrated exclusively at the ectopic position.

In order to evaluate whether the minimal *ori*Lyt by itself is sufficient to mediate replication of the HCMV genome, the sequences were inserted at the ectopic position of BAC pHG-Δ3.7, leading to pHG-2 and pHG-3, which differ in the orientation of the inserted minimal *ori*Lyt (Fig. 2). Characterization of the BAC DNAs with HpaI revealed the expected shift of an 8.1-kbp band to 11.2 kbp in pHG-2 and pHG-3 (Fig. 4A, lanes 4 and 5). After transfection of the mutant BACs into permissive human fibroblasts, single green cells were readily observed, but no plaques appeared on the cells (Fig. 4C), implying that no infectious virus can be recovered from pHG-2 and pHG-3, regardless of the orientation of the ectopically inserted minimal lytic origin. Thus, ectopic insertion of minimal *ori*Lyt is not sufficient to support replication of an HCMV genome in which complete *ori*Lyt has been deleted.

Since deletion of the complete *ori*Lyt may affect the promoter of the essential UL57 gene, we could not exclude the possibility that the observed phenotype of pHG-2 and pHG3 was the result of reduced UL57 expression. Therefore, minimal *ori*Lyt was also reinserted into pHG-Δ1.5, which lacks only the minimal lytic origin at the original position. However, the resulting BACs, pHG-4 and pHG-5 (Fig. 2 and 4), also did not lead to the development of plaques following transfection into human fibroblasts even after 4 weeks of incubation. These results suggest that the minimal lytic origin lacking the accessory flanking regions is not able to drive replication of the HCMV genome.

Reconstitution of infectious genomes after integration of complete *ori*Lyt at an ectopic position in CMV BACs with the origin deleted. Since the minimal lytic origin could not rescue the replication deficiency of pHG-Δ3.7 and pHG-Δ1.5, we tested whether ectopic insertion of the complete lytic origin

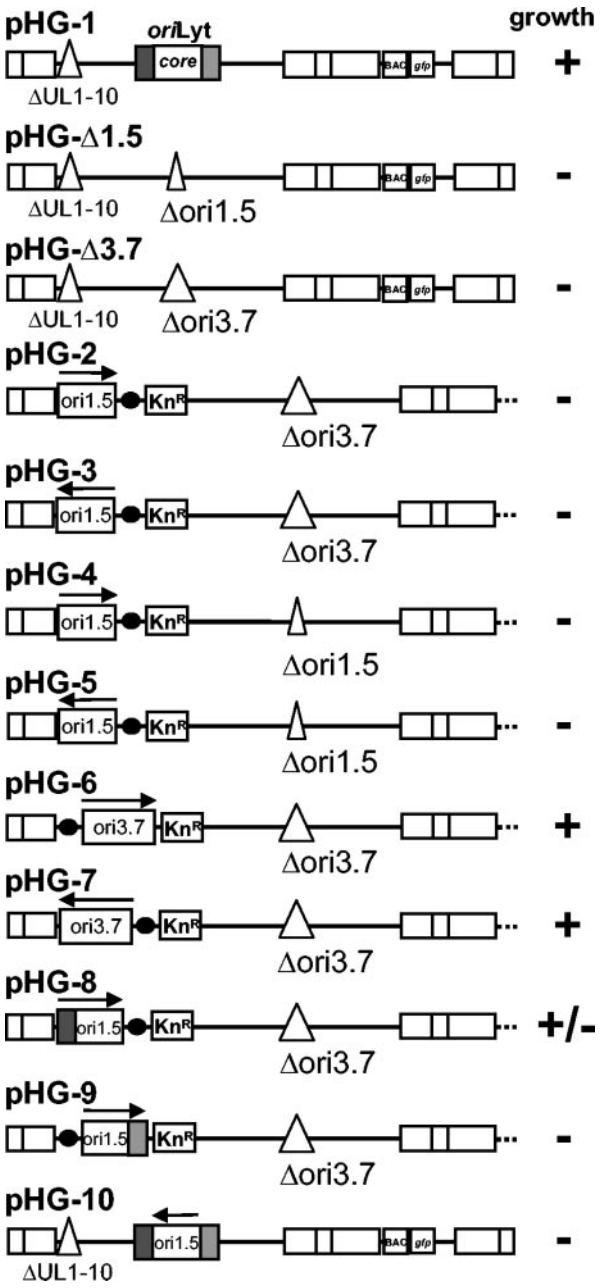

FIG. 2. Schematic overview of recombinant HCMV genomes generated and tested in this study. Shown are the parental BAC pHG-1 (top line) and the mutant BACs harboring a deletion of either the minimal (Δori1.5) or the complete (Δori3.7) lytic origin. Ectopic insertion of minimal *ori*Lyt (ori1.5), complete *ori*Lyt (ori3.7), minimal *ori*Lyt plus the left flanking region (dark grey), or minimal *ori*Lyt plus the right flanking region (light grey) is indicated by rectangles on the left-hand side of the genomes. The arrows reflect the respective orientations of the origin sequences. Also shown are the locations of the backbone (0.5 kbp; black dots) and the kanamycin resistance marker (1.0 kbp; Kn^R) of the shuttle vector. pHG-10 represents a genome carrying an inversion of the origin core at its original position. The genomes are not drawn to scale. The growth properties of the respective genomes are illustrated to the right: −, no plaque formation after transfection, +, plaque formation leading to complete CPE; +/−, formation of minifoci.

leads to replication-proficient HCMV genomes. The resulting genomes, pHG-6 and pHG-7, carry the complete *ori*Lyt at the ectopic position and lack these sequences at the original position (Fig. 2 and 4). Transfection of these genomes into permissive cells resulted in the formation of green fluorescent plaques (Fig. 4C) and in a complete cytopathic effect of the cultures ~2 weeks posttransfection. In order to analyze whether the genomes of the mutant viruses released from the transfected cells had preserved their original structures and to rule out the possibility that the lytic origin in the original region had been reconstituted, viral DNA was isolated from virions and examined by restriction analysis and Southern blotting. Figure 5A shows the BglII fragments of BACs pHG-6 and pHG-7 (lanes 2 and 4) and of the corresponding virus DNAs (lanes 3 and 5), as well as of the parental BAC pHG-1 (lane 1). In comparison to the circular DNA of the BACs, additional fragments (e.g., of 17.1 kbp) that originate from the terminal fragments of the linear viral DNA are visible in lanes 3 and 5. Probe p1, which is specific for the genomic region located to the left of the complete lytic origin (Fig. 5D), detected the expected 8.4-kbp band of the parental BAC pHG-1 (Fig. 5B, lane 1) and a 3.9-kbp band of pHG-6 and pHG-7, as well as of the corresponding viruses RV-pHG-6 and RV-pHG-7, which is indicative of the deletion of complete *ori*Lyt at the original position (Fig. 5B, lanes 2 to 5, and D). The blot was stripped and reprobed using fragment p2, specific for the lytic origin (Fig. 5D). As expected, hybridization with probe p2 revealed the same 8.4-kbp fragment of the parental BAC pHG-1 as did p1. In DNAs of the mutant BACs pHG-6 and pHG-7, a band of ~42 kbp was recognized, which corresponds to the expected fragment harboring the insertion of complete *ori*Lyt (Fig. 5C, lanes 2 and 4, and D, middle line). The DNA bands of the viruses RV-pHG-6 and RV-pHG-7 detected by probe p2 seemed slightly smaller (Fig. 5C, lanes 3 and 5), consistent with the expected size of ~29 kbp for the terminal fragments of two of the HCMV genome isomers (Fig. 5D, line 3). Two additional bands of ~36 and 42 kbp expected from the other genome isomers generated after inversion of the UL region could not be resolved on the gel shown in Fig. 5 but were readily detected after pulsed-field gel electrophoresis and hybridization with probe 2 (data not shown). Most importantly, hybridization of the probe to fragments with a size of 8.4 kbp or to any additional fragments was not found, ruling out the translocation of the ectopic *ori*Lyt back to its original position. Thus, we concluded that the mutant genomes were stable and that ectopic insertion of complete *ori*Lyt rescued the replication deficiency of genomes with *ori*Lyt deleted. The rescue was independent of the orientation of the reinserted origin sequences. These data also indicated that deletion of complete *ori*Lyt at the original position did not impair the expression of adjacent genes (e.g., UL57), at least not in a way that prevented growth of the viral mutants.

By integration of the sequences of the complete lytic origin at the ectopic position, we could also rescue the replication defect of pHG-Δ1.5, which lacks minimal *ori*Lyt at the original position (data not shown). The resulting genome readily produced plaques after transfection into permissive cells, finally leading to complete CPE. This result confirmed that the growth defect of pHG-Δ1.5 is caused by the deletion of mini-

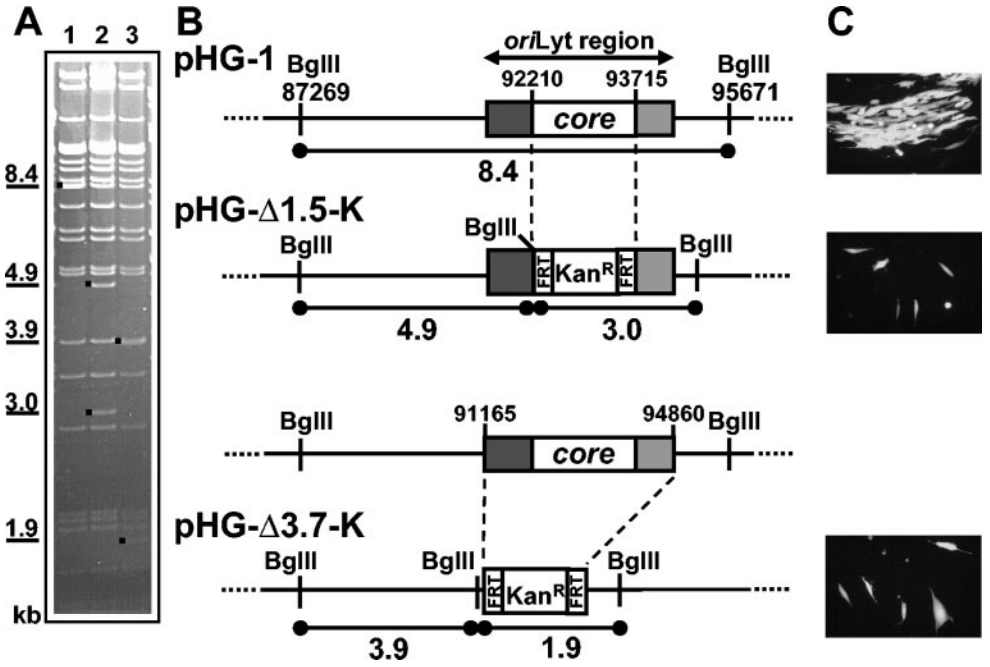

FIG. 3. Construction and analysis of HCMV genomes with deletion of the minimal or complete lytic origin. (A) Structural analysis of the parental BAC pHG-1 (lane 1) and mutant BACs pHG-Δ1.5K (lane 2) and pHG-Δ3.7K (lane 3) by treatment of the BAC DNA with BglII, followed by agarose gel electrophoresis and ethidium bromide staining. Relevant fragments are marked by black dots. Note that the 8.4-kbp band in lane 1 and the 3.9-kbp band in lane 3 are both comigrating with bands of similar size. (B) Schematic drawing of the genomes depicting the deletion of either minimal (core; pHG-Δ1.5-K) or complete (pHG-Δ3.7-K) *ori*Lyt. The sizes of fragments characteristic of the different BACs are indicated below each diagram. Dark grey, accessory region flanking the origin core to the left; light grey, accessory region flanking the core origin to the right; Kan^R, kanamycin resistance marker. (C) Human fibroblasts transfected with pHG-1, pHG-Δ1.5, or pHG-Δ3.7 analyzed 10 days posttransfection by UV light microscopy.

mal *ori*Lyt and is not due to any other potential mutation elsewhere in the genome.

Growth analysis of HCMV mutants with an ectopic *ori*Lyt. Following transfection of pHG-6 and pHG-7, we got the impression that the resulting plaques were slightly smaller than the plaques originating from the parental virus (Fig. 4C). Therefore, we compared the growth behavior of the mutant viruses RV-pHG-6 and RV-pHG-7 to that of the parental virus after infection of human foreskin fibroblasts with different viral doses. Following infection at a low MOI of 0.025 PFU/cell, the final titers obtained with mutant viruses RV-pHG-6 and RV-pHG-7 were reduced by ~1.5 orders of magnitude in comparison to the titers of RV-pHG-1 (Fig. 6A). However, the growth kinetics did not seem to be delayed. Similar results were obtained when cells were infected at a higher MOI of 1.5 PFU/cell (Fig. 6B). Thus, the HCMV mutants harboring the complete lytic origin at the ectopic position exhibited a growth defect in comparison to the parental virus.

To gain further insight into the growth behavior of RV-pHG-6 and RV-pHG-7, we analyzed the amplification of viral DNA in cells infected with either the parental virus, RV-pHG-1, or the mutant viruses RV-pHG-6 and RV-pHG-7. HFF were infected at an MOI of 0.02 PFU/cell, and total DNA was prepared from the infected cells at various times postinfection. The DNA samples were then analyzed by slot blotting, followed by hybridization with an HCMV-specific probe, and the signals obtained were quantified using a phosphorimager. As can be seen in Fig. 6C, comparable amounts of viral DNA were observed in fibroblasts infected with RV-pHG-1, RV-pHG-6, or RV-pHG-7. The mutants carrying the complete lytic origin at the ectopic position did not exhibit a defect in either the kinetics of DNA synthesis or final accumulation of viral DNA. We concluded from this result that the 3.7-kbp lytic origin inserted at the ectopic position comprises all of the information that is necessary to mediate efficient replication of the HCMV genome.

Role of the regions flanking the core replicator for *ori*Lyt activity. Since our data pointed to an important, or even essential, role of the sequences adjacent to minimal *ori*Lyt, we sought to dissect the influence of the right and the left flanking regions on *ori*Lyt activity. Therefore, minimal *ori*Lyt, together with either its right or left flanking region, was inserted at the ectopic position of pHG-Δ3.7, resulting in BACs pHG-8 and pHG-9 (Fig. 2 and 7). To evaluate the infectivity of these mutant genomes, the BACs were transfected into human fibroblasts, resulting in numerous green cells. Whereas these green cells remained single in pHG-9-transfected cultures, small foci of GFP-expressing cells were observed 3 weeks after transfection of pHG-8 (Fig. 7C). However, we did not observe the formation of plaques and the minifoci did not develop further into complete CPE as was observed with the parental BAC pHG-1. Therefore, we assume that pHG-8 is replication proficient, but the growth rate of the resulting virus is probably too low to promote the spread of the infection throughout the culture.

Having shown that the sequences flanking the *ori*Lyt core are crucial for origin function, we assessed whether the orientation of minimal *ori*Lyt relative to its flanking regions is rel-

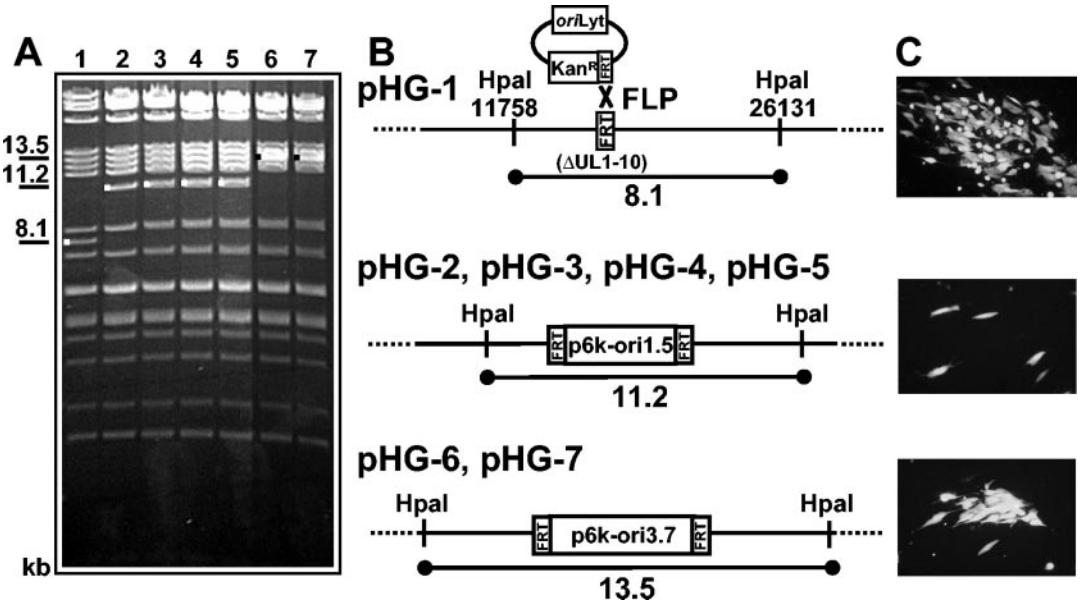

FIG. 4. Ectopic reinsertion of minimal or complete *ori*Lyt. (A) Restriction analysis with HpaI of pHG-1 (lane 1), recombinant BACs pHG-4 and pHG-5 (lanes 2 and 3) derived from pHG-Δ1.5 and harboring minimal *ori*Lyt in a different orientation at the ectopic position, and pHG-2, pHG-3 (lanes 4 and 5), and pHG-6 and pHG-7 (lanes 6 and 7) constructed by ectopic insertion of minimal *ori*Lyt and complete *ori*Lyt into pHG-Δ3.7 in different orientations. Relevant DNA bands are indicated. (B) Structure of the ectopic region before (pHG-1) and after insertion of the respective origin sequences (pHG-2 to pHG-7). Also depicted is the FLP-mediated integration of the shuttle plasmids into the FRT site at the ectopic position (top diagram). (C) Analysis of plaque formation on human fibroblasts transfected with either the parental or the respective mutant genomes. The micrographs show cell cultures at 10 days posttransfection with pHG-1 (top), pHG-2 (middle), and pHG-6 (bottom). Results virtually identical to those for pHG-2 and pHG-6 were obtained after transfection of pHG-3, pHG-4, or pHG-5, and of pHG-7, respectively.

evant. To this end, we inverted the 1.5-kbp origin core sequence at its original position. Starting with pHG-Δ1.5 missing the minimal *ori*Lyt, the core element was reintroduced in reverse orientation by a two-step mutagenesis procedure (see Materials and Methods for technical details). The DNAs of several BAC clones were analyzed by restriction analysis using BglII following mutagenesis (Fig. 8A). Two clones (clones 2 and 4) acquired the reversed minimal *ori*Lyt, as can be seen by

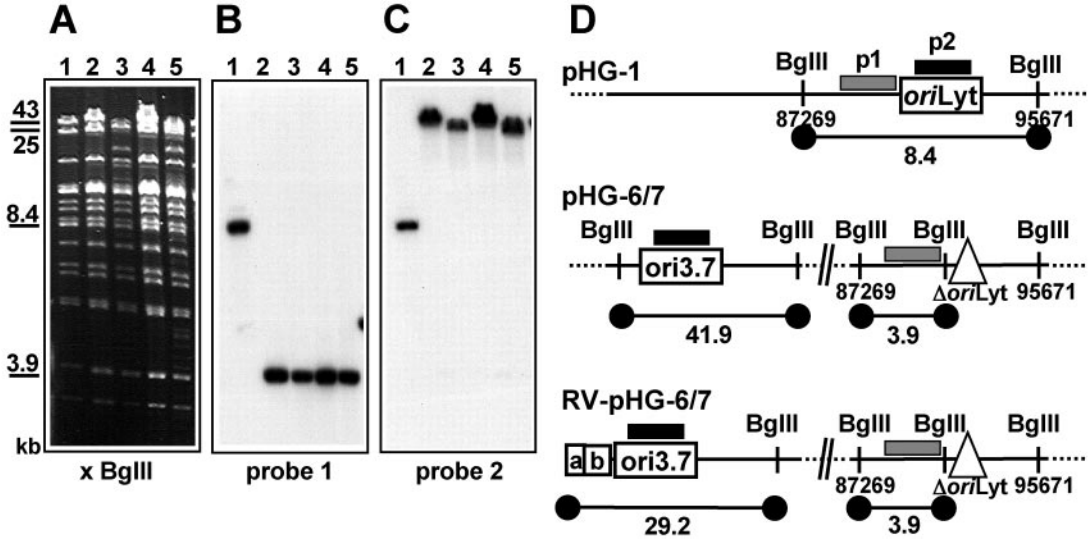

FIG. 5. Structural analysis of BACs pHG-6 and pHG-7 and of the genomes of the corresponding viruses RV-pHG-6 and RV-pHG-7. (A) Characterization of BAC DNAs (lane 1, pHG-1; lane 2, pHG-6; lane 4, pHG-7) and of viral DNAs (lane 3, RV-pHG-6; lane 5, RV-pHG-7) by BglII restriction analysis and agarose gel electrophoresis. The sizes of relevant DNA fragments are indicated. (B and C) Southern blot analysis with probes p1 and p2. (D) Schematic drawing of the predicted genomic regions representing the locations of *ori*Lyt in the parental (top scheme; pHG-1) and mutant (second scheme; pHG-6 and pHG-7) BACs and in the corresponding virus genomes (bottom scheme; RV-pHG-6 and RV-pHG-7). The locations of the probes used for hybridization are shown by black and grey bars, and the sizes of the hybridizing fragments are depicted below each diagram. The 29.2-kbp terminal fragment depicted in the bottom scheme arises from linear virus genomes with the UL region in the prototype orientation and represents one of three different fragments detected by probe p2 (see the text).

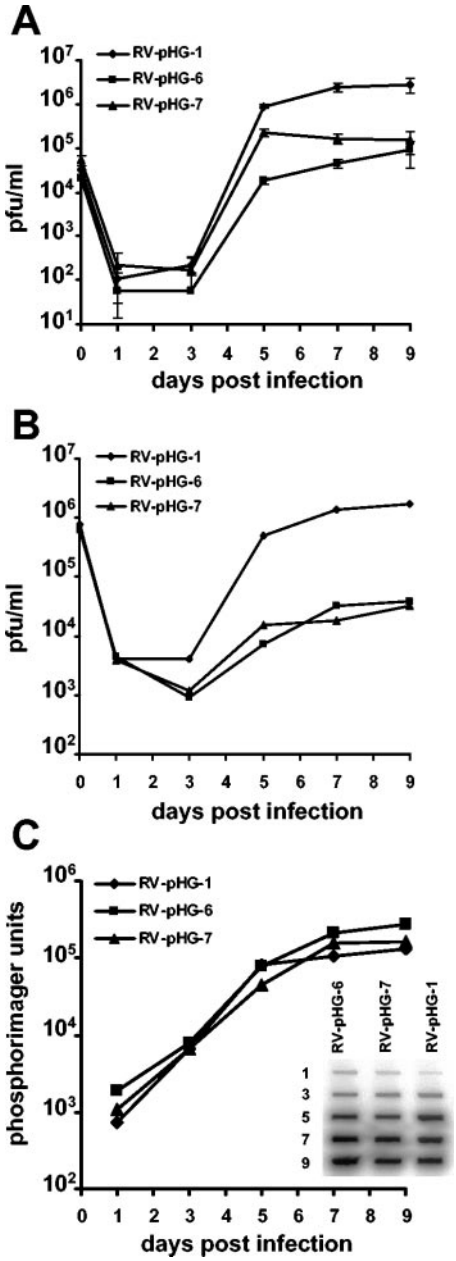

FIG. 6. Growth analysis of the parental virus RV-pHG-1 and of the mutant viruses RV-pHG-6 and RV-pHG-7. (A and B) HFF were infected at an MOI of 0.025 (A) or 1.5 (B), and at the indicated times postinfection, supernatants of the infected cultures were analyzed for infectious virus by plaque assay. Each data point represents the average of three independent wells. Titers at day zero represent input inocula. (C) Amplification of viral DNA after infection. HFF were infected at an MOI of 0.02 PFU/cell, and total DNA was prepared on days 1, 3, 5, 7, and 9 postinfection. The DNAs were assayed by slot blotting using an HCMV-specific probe, followed by quantification of the radioactivity using a phosphorimager.

the presence of an additional 8.4-kbp fragment and the lack of the 4.9- and 2.0-kbp bands (Fig. 8A, lanes 2 and 4). In order to test the consequences of the inversion, pHG-10 clones 2 and 4 were transfected into permissive fibroblasts, leading to numerous green fluorescent cells. However, only single green cells could be observed during a 4-week incubation period, but no plaques appeared on the cell monolayers (Fig. 8B). This experiment implied that the appropriate orientation of the minimal lytic origin with respect to its flanking regions is crucial for origin function.

DISCUSSSION

In this study, we investigated the sequence requirements of the described lytic origin of human cytomegalovirus (*ori*Lyt) in the context of the viral genome. To our knowledge, this is the first report of the intentional generation of recombinant HCMV genomes and viruses carrying mutations of *ori*Lyt. Our results provide evidence that the HCMV genome of strain AD169 contains only one lytic origin, because HCMV genomes with deletion of either minimal or complete *ori*Lyt were not infectious. Furthermore, we show that the 1.5-kbp region designated the HCMV core is not sufficient to drive replication of HCMV, since ectopic insertion of this sequence into a nonessential region of the viral genome did not rescue the replication-deficient phenotype of the genomes with the origin deleted. Rather, accessory sequences flanking minimal *ori*Lyt are needed for replication of the HCMV genome, as insertion of the complete lytic origin (encompassing these regions) readily resulted in infectious viral progeny. The observation that genomes with a reinsertion of the origin core together with its left flanking region gave rise to the formation of minifoci whereas genomes harboring the origin core plus the sequences to the right did not suggested the presence of an essential element within the left flanking region. Notably, correct orientation of the origin core relative to its auxiliary regions was crucial for replicator function.

One of the biggest advantages of the BAC-based mutagenesis of herpesviruses is the fact that construction and testing of mutant genomes are separate steps. This allowed us to manipulate HCMV *ori*Lyt, a *cis*-active element of the genome considered to be essential for viral replication. The BAC technique enabled us to perform several rounds of mutagenesis on the very same genome, independent of the phenotype of the intermediates, as well as to construct replication-deficient CMV genomes that harbor lethal mutations in essential elements of *ori*Lyt.

Deletion of minimal or complete *ori*Lyt led to replication deficiency of the resulting genomes, indicating that the deleted sequences are essential and that other elements of the genome cannot substitute for them. These findings suggest that in contrast to human alpha- and gamma-herpesviruses (4, 26, 46, 57), the HCMV genome contains only one lytic origin. This might be a unique feature of beta-herpesviruses, since so far only one single origin of replication has also been described for simian, rat, and mouse CMV, as well as for human herpesvirus types 6 and 7 (2, 23, 38, 52, 53). Of course, this hypothesis requires experimental examination. Our results apply only to the laboratory strain AD169, and due to the fact that clinical isolates of HCMV contain additional DNA sequences (12), we cannot rule out the possibility that such CMV strains harbor more than one lytic origin. Whether the presence of several lytic origins provides a replication advantage remains elusive, because deletion of *oriL* or both copies of *oriS* of HSV had only marginal effects on viral growth (30, 44).

The final evidence that the replication deficiency of the

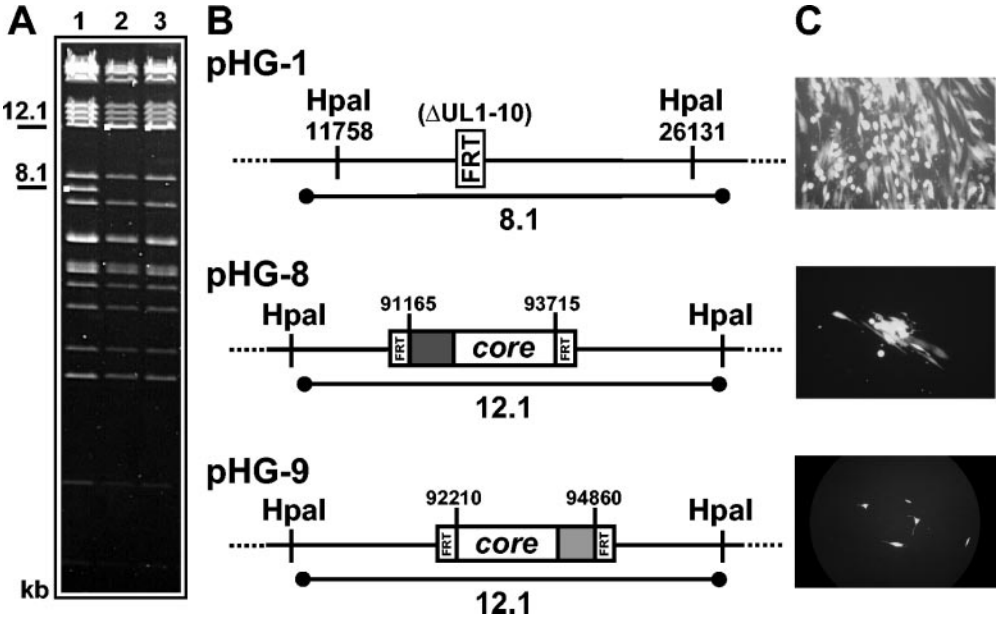

FIG. 7. Ectopic insertion of the origin core together with either its left or right flanking region. (A) DNAs of pHG-1, pHG-8, and pHG-9 (lanes 1 to 3) were cut with HpaI and separated on a 0.5% agarose gel, followed by ethidium bromide staining. (B) Structure of pHG-8 (middle line) and pHG-9 (bottom line) after FLP-mediated insertion of the indicated origin sequences. (C) HFF cells transfected with pHG-1, pHG-8, or pHG-9 were analyzed for plaque formation 3 weeks after transfection.

genomes with the origin deleted is due to the deletion of *ori*Lyt is technically difficult to supply. The phenotype could also result from the impairment of another essential element, e.g., the ORF of an essential gene located in the same region, and the genetic elements may be physically inseparable. For the following reasons we consider this rather unlikely. (i) Recent reevaluation of the HCMV coding potential based on comparison with the genetic information for the closely related chimpanzee CMV strongly suggested that most, if not all, of the previously annotated ORFs within the region encompassing *ori*Lyt do not encode functional proteins (20, 43). (ii) Meanwhile, some of the ORFs have indeed been shown to be nonessential (24). (iii) Sequence variability in the *ori*Lyt region was observed, a fact that is hardly compatible with the potential to

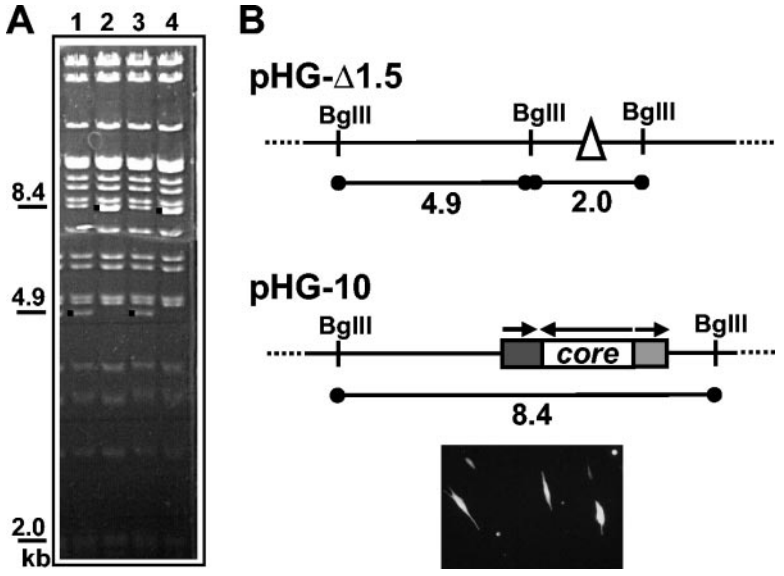

FIG. 8. Inversion of the origin core at its original position in the HCMV genome. (A) Following mutagenesis, BAC DNA was isolated from several clones and checked for the presence of fragments indicative of the mutant genomes by restriction analysis with BglII. Lanes 1 and 3, DNAs of BAC clones with a structure identical to that of parental pHG-Δ1.5. Lanes 2 and 4, DNAs of BACs that acquired the origin core in inverted orientation. DNA bands characteristic of the different BACs are marked by black dots. (B) Structure of the HCMV *ori*Lyt regions of pHG-Δ1.5 harboring a deletion of the origin core and of pHG-10 carrying the origin core in inverted orientation. Shown below are human fibroblasts transfected with pHG-10 3 weeks posttransfection.

encode proteins (14, 15, 32, 45). (iv) In agreement with the phenotypes of the genomes with *ori*Lyt deleted described in this study, at least three regions within minimal *ori*Lyt were found to be indispensable for origin function on the basis of transient replication assays (1, 37, 45, 59).

Assuming that *ori*Lyt does essentially act in *cis* by providing an initiation site for the replication machinery, we tested whether the defect of the genomes with the origin deleted could be rescued by ectopic reinsertion of these sequence elements. This was indeed the case for complete *ori*Lyt, whereas minimal *ori*Lyt was not sufficient. In view of the low replication activity of minimal *ori*Lyt that was determined in transient transfection experiments using the subcloned element (1, 37, 59), the observed insufficiency of minimal *ori*Lyt to support replication of the complete genome was perhaps not surprising. The results indicate, however, that the *ori*Lyt of HCMV represents more than just an initiation site for replication. This may be in contrast to the origins of alpha-herpesviruses that span only ≤150 bp and basically provide the start site for DNA replication and binding sites for the origin-binding protein (46). Possibly, the lack of an origin-binding protein of HCMV was the driving force for the development of such a large and complex origin and of *cis*-active sequence elements that could either substitute for the function of an origin-binding protein or support initiation of DNA replication by an alternative mechanism.

Rescue of the replication-deficient phenotype by reinsertion of the complete *ori*Lyt demonstrated that these sequences encompass all elements necessary to initiate DNA replication at the ectopic position. Moreover, these results show that the activity of this important *cis*-active element is neither position nor orientation dependent. The mutants were not deficient either in the kinetics of viral DNA synthesis or in the DNA yields. These findings also strongly suggest that the mutants were not impaired in the expression of transcripts that are essential for the initiation and progression of viral DNA replication, e.g., the smallest replicator transcript (SRT), v-RNA-2, or the UL57 RNA encoding the single-stranded DNA binding protein. The 5′ ends of the UL57 transcripts were precisely mapped in a recent study (33). Although the UL57 promoter may extend considerably upstream, it is extremely unlikely that the growth defect of the genomes with a deletion of minimal *ori*Lyt was caused by an impairment of UL57 expression, especially since pHG-Δ1.5 could be rescued by ectopic insertion of the complete *ori*Lyt. Since the left boundary (nt 91165) of the complete *ori*Lyt as used in this study and the most distal transcription start site of the UL57 RNA (nt 91138), as well as the candidate promoter sequences (33), are located in close proximity, one cannot completely rule out an effect on UL57 expression in the mutants carrying a deletion of complete *ori*Lyt. However, given the pivotal role of the UL57 protein (single-stranded DNA binding protein) for replication of HCMV DNA, impaired UL57 expression would hardly be compatible with the unaltered DNA synthesis displayed by the mutants RV-pHG-6 and RV-pHG-7.

In view of the results of the DNA synthesis, the reduced viral yields of these mutants are difficult to explain. One hypothesis is that the promoter-enhancer elements in the origin sequences may have an effect on the expression of genes with important functions in the infection cycle that are not involved in DNA replication. Further work will be needed to define the reasons for the impaired growth properties of these mutants.

Having shown that the accessory regions of minimal *ori*Lyt are essential for replication of the genome, we investigated whether one of the flanking regions may be dispensable. Transfection of genomes containing minimal *ori*Lyt plus the left flanking region resulted in small groups of GFP-expressing cells. We called these clusters of green cells minifoci, because they did not develop into typical plaques and complete CPE did not occur. Although the experimental evidence is difficult to obtain, we assume that the pHG-8 genome is able to replicate in the initially transfected cells, albeit at very low efficiency. The resulting progeny are probably able to spread to the neighboring cells, but then the infection terminates because either the growth rate of the virus is too low or an antiviral state is induced in the surrounding cells, e.g., by cytokines, which cannot be overcome.

Genomes with minimal *ori*Lyt plus the right flanking region did not lead to plaques or to minifoci, suggesting that an essential element is located to the left of minimal *ori*Lyt. In fact, the precise boundaries of HCMV *ori*Lyt have been a matter of debate. Masse et al. specified that sequences flanking the minimal *ori*Lyt contribute to efficient replication but are not essential for origin function (37). Zhu et al. mapped the core of *ori*Lyt to a region encompassing nt 91751 to 93299 (59), which includes part of the left flanking sequence of minimal *ori*Lyt as defined by Masse et al. According to Zhu et al., the core replicator is necessary, but not sufficient, to drive DNA replication in transient assays (59). The fact that ectopic insertion of the left accessory sequence plus minimal *ori*Lyt led to replication-competent genomes underlines its importance for origin function. Since the resulting virus displayed an extremely reduced growth capacity, one has to conclude that the right flanking sequences also play an important, yet not essential, role for replicator activity.

The accessory regions of HCMV *ori*Lyt contain numerous binding sites for a series of transcription factors. Thus, one may speculate that these regions mediate their effect on the core replicator in a manner similar to that of enhancer sequences on promoter regions. If this is true, one would expect that they can act in an orientation- and distance-independent manner. Since inversion of minimal *ori*Lyt resulted in a replication-incompetent genome, the hypothesis has to be discarded. Instead, the transcription factor binding sites rather may represent promoter elements, and indeed, a number of transcripts arise from the HCMV origin region, including the SRT, vRNA-1, and vRNA-2 (Fig. 1) (29, 45). Origin-associated transcripts were also identified in other viral, as well as cellular, origins (22, 54). Possibly, they have no messenger function but rather participate either in the replication process itself, e.g., by serving as a primer for the DNA polymerase, or in a more indirect manner, e.g., by inducing changes in the superhelical structure of the DNA template. Interestingly, all known transcripts from the HCMV origin region extend from right to left in the prototype arrangement of the viral genome. If we assume that these transcripts have an important role in the initiation of viral DNA replication, one can understand that inversion of minimal *ori*Lyt and the concomitant change in the direction of transcription of the vRNA-2 and SRT genes will disrupt origin function.

We report in this study the first HCMV recombinants with mutations in *ori*Lyt. By using the highly convenient FRT/FLP recombination system, we established a fast and easy way to reinsert *ori*Lyt sequences into a nonessential ectopic region of the HCMV genome in order to dissect the roles of *cis*-acting elements of *ori*Lyt. The BAC-based mutagenesis techniques also allowed us to manipulate the origin sequences at their original position. In further work, we can evaluate the functions of specific origin elements, which were defined in transient replication assays, in the context of the viral genome to deepen our understanding of the complex mechanism of HCMV DNA replication.

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