Identification of the Lytic Origin of DNA Replication in Human Cytomegalovirus by a Novel Approach Utilizing Ganciclovir-Induced Chain Termination

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Received 3 July 1990/Accepted 3 September 1990

Infection with human cytomegalovirus in the presence of the antiviral nucleotide analog ganciclovir results in continuing low-level viral DNA synthesis and the accumulation of relatively small fragments of double-stranded progeny DNA. These fragments consistently proved to represent amplification of sequences from only one small section of the viral genome (*EcoRI*-V) lying near the center of the unique L segment. Further mapping revealed that the viral sequences represented in these fragments occurred in gradients of abundance that decreased in both directions from a point near 0.35 to 0.4 map unit. The proportion of amplified sequences increased with both time after infection and dosage of ganciclovir used. We conclude that the primary lytic cycle replication origin of human cytomegalovirus lies within a 3- to 4-kb region immediately upstream and to the right of the promoter for the single-stranded DNA-binding protein (DB140). The amplified origin-containing DNA molecules appeared to arise by continuing rounds of bidirectional initiation on truncated fragments of the genome that were generated as a result of chain termination effects induced by the incorporation of ganciclovir into the viral DNA. Inspection of the DNA sequence in the vicinity of *ori*-Lyt revealed a large complex upstream region that may be a noncoding intergenic domain and that bears no homology to any previously described herpesvirus origin. This 2.5-kb region includes many duplicated and inverted sequences, together with consensus CRE/ATF and other transcription factor-binding sites, and an interesting set of 23 copies of an interspersed decamer consensus element AAAACACCGT that is also conserved at the equivalent locus in simian cytomegalovirus. This work represents the first identification of an origin domain in a cytomegalovirus genome and is the first demonstration of a bidirectional mechanism for any herpesvirus lytic cycle origin.

Both primary and reactivated infections with human cytomegalovirus (HCMV) can lead to serious clinical consequences in acquired immunodeficiency syndrome or otherwise immunocompromised patients. In recent years, the nucleotide analog ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl) guanine (DHPG)] has received considerable attention as the first antiviral agent that shows some efficacy against HCMV (2). While establishing a DNA hybridization assay for studying the effects of DHPG on cytomegalovirus (CMV) replication in cell culture systems, we observed that, contrary to expectations, newly synthesized viral DNA continued to accumulate in the nucleus at DHPG doses that totally inhibited virion production (F. M. Hamzeh and P. S. Lietman, submitted for publication). Further analysis, as described here, has revealed that this DNA is of relatively low molecular weight and represents amplification of only a limited portion of the viral genome.

The locations of individual defined replication origins in mammalian genomes have proved extremely difficult to define and characterize (18). Therefore, the origins for initiation of DNA synthesis in DNA viruses have attracted much attention as model systems. A great deal is known about the detailed mechanism of these events in the relatively small and specialized papovavirus and adenovirus systems and also in the highly lytic herpes simplex virus (HSV). The situation prevailing in some of the more cell-

associated herpesviruses and papillomaviruses, which appear capable of replicating in a latent state in close coordination with cellular replication controls, is expected to provide additional insights.

In Simian Virus 40 (SV40), adenovirus, and HSV, relatively short minimal origin DNA sequences are involved, although in HSV essentially the same sequences are duplicated at three distinct loci, *ori*-L, *ori*-S1, and *ori*-S2 (6, 16, 30, 41, 42, 45). Each of these three viruses also encodes an origin-specific DNA-binding initiator protein (e.g., SV40 large T antigen, adenovirus terminal protein, and HSV UL9). UL9 binds to consensus GTTCGCAC sequences located on both arms of 47-bp (*ori*-S) or 142-bp (*ori*-L) palindromic sequences, which also have an A+T-rich central core (9, 25, 33, 44). HSV replicates in an extremely wide range of host cell types and encodes six additional viral polypeptides necessary for replication, including a delta-like DNA polymerase and polymerase-associated protein, a single-strand DNA-binding protein, and primase and helicase activities (3, 7, 32, 46). Large T antigen also possesses ATPase and unwindase activities, and adenovirus encodes its own DNA polymerase and single-stranded DNA-binding protein, but otherwise replication in vitro of both SV40 and adenovirus DNA depends upon several additional and essential host cell proteins (4).

In the case of Epstein-Barr virus (EBV), two distinct replication origins have been identified by DNA transfection procedures. One operates during latency and is responsible for maintenance of circular plasmids in B lymphocytes

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(*ori-P*). Plasmid replication is coupled to the cell cycle and utilizes the cellular DNA polymerase and a single origin-specific viral DNA-binding protein, EBNA-1 (36, 48, 49). There are 24 binding sites for EBNA-1 in the relatively large (1.5-kb) *ori-P* region: 4 in an initiator domain with dyad symmetry and 20 arranged as 30-bp tandem repeats in a distinct terminator domain (5, 14, 34, 47). In contrast, uncoupled lytic-cycle replication in EBV requires a duplicated 1,000-bp region referred to previously as DSL and DSR (17). These two *ori-Lyt* sites contain many repetitive and palindromic features and include an enhancer domain with binding sites for several cellular proteins and for the EBV Zta transcriptional *trans* activator (10, 19, 28; Y.-N. Chang, G. S. Hayward, and S. D. Hayward, unpublished data).

Each of the known viral lytic DNA replication origins appears to contain at least one recognizable A+T-rich initial melting site and lies within a divergent intergenic noncoding region adjacent to or intermingled with transcriptional control elements for key immediate-early promoters or replication-related genes. The physical structures of replicating SV40, adenovirus, and HSV DNAs are dramatically different from one another. SV40 proceeds primarily through a circular "Cairns" or "theta" replicative intermediate, whereas adenovirus utilizes a single-strand displacement mechanism proceeding from either end of the linear genome. Herpesviruses all produce fast-sedimenting multigenomic concatemeric intermediates and have been proposed to first circularize their input linear genomes and then replicate by a "rolling-circle" process (13, 20, 21, 37). However, the only direct evidence for this model comes from the well-defined tandem-repeat structure of defective HSV DNA species that accumulate at high multiplicities of infection (11). These defective DNA populations retain only short segments of the viral genome from adjacent to one or the other of the origins and include terminal packaging sequences. Their structure provided the initial evidence for the map location of the *ori-S* and *ori-L* origins in HSV (6, 12, 22, 29).

No information is available as yet about the molecular events in DNA replication of the 240-kb genome of HCMV, which produces efficient permissive lytic cycle infection in cell culture only in diploid human fibroblasts. However, it is known that newly synthesized intracellular replicating HCMV DNA, like that of HSV and EBV, contains joined termini (27), and there is some evidence in murine CMV for circularization of input parental genomes (31). Because of previously unsuccessful attempts to identify the origin of HCMV replication by the traditional DNA cotransfection and superinfection assays pioneered by Stow (44) and by Challberg (3), the alternative approach described here of chain termination mapping induced by DHPG has proved valuable.

(This study represents work submitted by F.M.H. in partial fulfillment of requirements for his Ph.D. degree in Pharmacology and Molecular Sciences from The Johns Hopkins University.)

MATERIALS AND METHODS

Cells and virus. Human embryonic lung fibroblast (MRC-5) cells were obtained from the American Type Culture Collection (Rockville, Md.). The cells were maintained and passaged in Dulbecco minimal essential medium (DMEM) containing 10% fetal bovine serum (GIBCO). The cells were plated in either six-well plates or individual 100-mm dishes. HCMV (Towne) was plaque purified and

maintained by passaging at a low multiplicity of infection (0.01 to 0.1 PFU/cell) in the above cells (27). The virus was stored in DMEM at -80°C . Cell cultures were infected when they reached confluence. The virus was added at the designated multiplicity of infection in DMEM containing 3% heat-inactivated fetal bovine serum. The cultures were incubated for 90 min at 37°C in a 5% CO_2 incubator for adsorption of the virus. The adsorption medium was then aspirated, and the monolayer was washed twice with phosphate-buffered saline (0.1 ml/cm² of growth surface). Fresh medium was added with or without DHPG at the designated concentrations. DHPG was obtained originally from the Burroughs Wellcome Co., Research Triangle Park, N.C., and can now be obtained from Syntex, San Francisco, Calif.

Virion DNA preparation. MRC-5 cells were plated in a 75-cm² flask. The cells were infected with HCMV at a low multiplicity of infection as described above. On day 5 after infection, the medium was replaced with fresh medium for 3 days. On day 8 after infection, the medium was transferred to a sterile tube and then centrifuged at 5,000 rpm for 15 min to remove cellular debris. The supernatant fluid was collected and transferred to a new sterile tube. The virus was pelleted by centrifuging the above supernatant fluid in an RC-2B Sorvall centrifuge at 18,000 rpm for 2 h at 4°C . The virus pellet was digested with proteinase K, and the virion DNA was extracted with phenol as described below.

Intracellular DNA extraction. At specific time points, infected monolayers were washed twice with phosphate-buffered saline and then either scraped directly into 2.5 ml of phosphate-buffered saline or collected after trypsinization. After cells were pelleted by centrifugation at 2,000 rpm at 4°C for 5 min, they were suspended in phosphate-buffered saline and counted and then stored at -80°C for dot blot hybridization without nucleic acid extraction. For larger-scale DNA extraction, the cells were suspended in lysis buffer (1.0% Sarkosyl, 0.01 M EDTA, 0.01 M Tris hydrochloride [pH 7.6]). Proteinase K (preincubated for 30 min at 37°C) was added to a final concentration of 200 $\mu\text{g}/\text{ml}$. The lysate was then incubated at 37°C for 30 min. Proteins were extracted by successive cycles of phenol, phenol-chloroform-isoamyl alcohol (50:48:2), and chloroform-isoamyl alcohol (48:2). Nucleic acids were precipitated with ethanol at -20°C and then suspended in TE buffer (0.01 M Tris hydrochloride 0.01 M EDTA [pH 7.4]). Total DNA concentration was determined, and the samples were stored at -20°C for further analysis.

Viral DNA synthesis as measured by dot blot DNA-DNA hybridization. Cells ($2 \times 10^5/\text{ml}$) were prepared for dot blot hybridization without DNA extraction by adding sodium hydroxide to a final concentration of 0.5 M and incubating for 1 h at 37°C . At the end of the incubation period, the samples were neutralized with an equal volume of 3 M sodium acetate (pH 5.2). The samples were applied to nylon membranes (Biodyn A; Pall Corp.) under vacuum with a 96-well apparatus (Schleicher & Schuell, Inc.). The membranes were dried at 80°C for 3 h and then stored in a closed plastic bag until hybridization. Prehybridization was carried out in a sealed bag in a solution containing $6 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.4]), $5 \times$ Denhardt solution (0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.1% bovine serum albumin), 0.2% sodium dodecyl sulfate (SDS), and 100 μg of salmon sperm DNA per ml (all reagents from Sigma). After prehybridization for 2 h at 67°C , the membranes were hybridized for 24 h at 67°C in a mixture of the same composition as prehybridization buffer, but with the addition of 10^7 cpm of a radioactive HCMV DNA probe.

The probe was labeled with [32 P]dCTP by nick translation with a commercial nick translation kit (Bethesda Research Laboratories) or by random priming as recommended by the manufacturer of the Oligolabeling Kit (Pharmacia). At the end of the hybridization period, each membrane was washed in at least 250 ml of each of the following solutions: 0.5% SDS–2 $\times$ SSC for 5 min, 0.1% SDS–2 $\times$ SSC for 15 min, 0.5% SDS–0.1 $\times$ SSC at 65°C for 2 h with gentle agitation, and finally 0.5% SDS–0.1 $\times$ SSC for 30 min at 65°C. The membranes were dried at 80°C for 2 h and then exposed to X-ray film (Kodak X-Omat). Finally, individual dots were cut out, and the radioactivity associated with the probe was quantified by scintillation counting in Fluorosol (National Diagnostics) with a Beckman LS-233 scintillation spectrometer.

Sucrose gradient centrifugation of DNA. Total cellular DNA samples extracted from DHPG-treated and untreated HCMV-infected cultures were layered at the top of linear 10 to 30% (wt/vol) sucrose gradients prepared in TBS buffer (0.15 M NaCl, 0.05 M Tris hydrochloride, 0.01 M EDTA [pH 7.4]). The gradients were centrifuged in a Beckman SW41 rotor in an OTD-50 Sorvall ultracentrifuge at 35,000 rpm for 3 h at 18°C. Fractions of 0.5 ml each were collected from the top of the gradient. The viral DNA content of each fraction was determined by dot blot DNA-DNA hybridization with the designated probe.

Southern blot analysis. The fractions from the sucrose gradient were combined as indicated below for each experiment. The combined fractions were dialyzed against TBS buffer (250 ml) with three changes of the dialysis buffer. Finally, the DNA was precipitated with ethanol at –20°C and suspended in TE buffer for storage at –20°C. The DNA samples were cleaved with the designated restriction endonuclease and then electrophoresed in agarose gels and transferred to a nylon membrane (39). The DNA from the top of the gradient, representing the slowly sedimenting molecules, was 32 P labeled as above and hybridized to the Southern blot membranes by using the procedure and washing conditions described above.

Cloned DNA probes. The individual *Bam*HI and *Hind*III fragments from HCMV (Towne) virion DNA described by LaFemina and Hayward (26), Thomsen and Stinski (43), and D'Aquila et al. (8) were cloned in plasmid pBR322 in an *Escherichia coli* HB101 (*rec*) host. The resulting plasmids used in this work were pRL3 (*Bam*HI–C, 16.2 kb), pRL11 (*Bam*HI–K, 8.5 kb), pRL16 (*Bam*HI–Q, 6.1 kb), pRL104 (*Hind*III–D, 20.3 kb), and pRL113 (*Hind*III–M, 8.9 kb). The map locations of these fragments are shown in Fig. 1b and 3b. Plasmid DNA was purified by CsCl-ethidium bromide density gradient centrifugation followed by ethanol precipitation and dialysis.

Mitochondrial DNA probe. The mitochondrial DNA probe was prepared from human blood platelets as described by Schuster et al. (38). In summary, the blood platelets were centrifuged at 200 $\times g$ for 10 min at room temperature to remove contaminating cells. The platelets were pelleted at 750 $\times g$ for 20 min and digested with proteinase K; then the nucleic acids were extracted with phenol. RNA was removed by digestion with DNase-free RNase (0.1 mg/ml). The remaining DNA was digested with *Bam*HI and subjected to electrophoresis in 0.8% low-melting-temperature agarose. The single 16.4-kb mitochondrial fragment was recovered and then labeled with [32 P]dCTP by the random priming procedure described above.

FIG. 1. Reduced size and limited sequence complexity of HCMV DNA in DHPG-treated cultures: sucrose gradient sedimentation analysis of total intracellular or virion DNA extracted 96 h after infection in the presence or absence of DHPG. The distance sedimented increases from left to right. The amount of viral DNA in each fraction was detected by dot blot filter hybridization with cloned HCMV fragment DNA probes. (A) Control DNA extracted from purified virions; *Bam*HI–C probe. (B) Intracellular DNA from cultures treated with DHPG at 0 (○), 1 (●), or 10 (Δ) μg/ml; *Bam*HI–C probe. (C) Intracellular DNA from HCMV-infected cells analyzed with probes from different regions across the genome: *Bam*HI–K (○), *Hind*III–M (●), and *Bam*HI–Q (Δ). Each fraction is plotted as a ratio (percentage) of the radioactivity hybridizing to DNA from a culture treated with DHPG at 10 μg/ml relative to that obtained with DNA from a parallel untreated culture.

RESULTS

Short DNA fragments that accumulate in the presence of DHPG represent selectively amplified sequences. Treatment of HCMV-infected cells with DHPG inhibits the production of infectious progeny virus as measured by a plaque reduction assay but permits continued synthesis of viral DNA detectable by dot blot hybridization with a cloned HCMV *Bam*HI–C DNA probe (Hamzeh and Lietman, submitted). The HCMV DNA that accumulates consists of short incomplete viral DNA fragments rather than complete genomes as judged by sucrose gradient sedimentation properties. We hypothesized that these short DNA fragments could have resulted from chain termination, which might result in the selective accumulation of viral DNA sequences from regions of the genome that are close to the origin or origins of replication. To test this hypothesis, we designed several

FIG. 2. Physical map locations in the intact HCMV (Towne) genome of the four DNA probes used in these experiments (solid bars). Complete restriction enzyme cleavage maps for *Hind*III, *Bam*HI, and *Eco*RI are modified from the data of LaFemina and Hayward (26), Kemble et al. (23), and D'Aquila et al. (18). Open bars indicate the inverted repeats encompassing the U_L and U_S segments.

experiments to ask whether specific regions of the HCMV genome are selectively represented in these short fragments.

In the first experiment, intracellular DNA samples from HCMV (Towne)-infected MRC-5 cells grown either in the absence of DHPG or the presence of 1 or 10 μ g of DHPG per ml were extracted and fractionated by sedimentation through sucrose density gradients. The collected fractions were analyzed for viral DNA content by dot blot hybridization with the same *Bam*HI-C probe (pRL3) used previously. This analysis revealed both a reduction in total yield of viral DNA and a shift from rapidly sedimenting virion-sized DNA molecules to slowly sedimenting short DNA fragments (Fig. 1A and B). However, different results were obtained when probes from other sections of the HCMV genome were used. For example, hybridization with a *Bam*HI-K probe to DNA samples from across the sedimentation profile of intracellular DNA grown in cells infected in the presence of 10 μ g of DHPG per ml gave a similar result to that with the *Bam*HI-C probe, but neither the *Hind*III-M nor *Bam*HI-Q probe detected any significant amounts of homologous viral sequences in any portion of the gradient (Fig. 1C). The *Bam*HI C and K fragments map adjacent to one another near the center of the unique L segment of HCMV (Towne) DNA, whereas *Bam*HI-Q lies in the unique S segment and *Hind*III-M comes from near the left-hand end of the unique L segment (Fig. 2). Therefore, although alternative explanations were possible, it seemed likely that the DHPG-generated fragments represented selective amplification of sequences from only a limited portion of the viral genome.

In a second set of experiments, enrichment of *Bam*HI-K sequences compared with *Bam*HI-Q sequences was studied as a function of both the concentration of DHPG used and the length of time of the treatment. Both dot blot and Southern blot experiments were carried out with total unfractionated intracellular DNA. Measurement of the ratio of remaining sequences that were complementary to the *Bam*HI-K or *Bam*HI-Q probes at 1 and 25 μ g/ml of DHPG, relative to untreated controls, showed that the fraction of *Bam*HI-Q sequences present fell to only 35 and 2%, respectively, of that in the untreated controls within 2 days after infection (Fig. 3A). However, the relative levels of *Bam*HI-K sequences never fell beyond 60 and 8%, respectively, at the two doses and recovered to 90 and 23%, respectively, at 4 days. Since there was a considerable increase in total HCMV DNA at 4 days compared with that at 2 days in the untreated control samples, these data indicated that there was a net gain in total *Bam*HI-K

sequences and that this part of the genome is selectively replicated at increasingly higher rates than are the *Bam*HI-Q sequences in the presence of greater concentrations of DHPG.

Southern blot hybridization to *Bam*HI and *Eco*RI digests of total intracellular DNA prepared at 4 days after infection in the presence of increasing doses of DHPG confirmed that these probes were, indeed, hybridizing to the correct viral DNA fragments and also that the phenomenon can be demonstrated at the level of the abundance of specific intact viral DNA fragments (Fig. 3B). Moreover, since *Bam*HI-K is a larger fragment than *Bam*HI-Q, the relative increase in *Bam*HI-K sequences clearly involves an amplification event and is not simply a matter of random degradation.

Identification of the viral DNA locus that is amplified in the presence of DHPG. The peak fractions containing most of the slowly sedimenting short DNA fragments from a DHPG-treated culture (Fig. 1B, fractions 3 to 8) were combined and digested with either *Bam*HI or *Eco*RI restriction enzyme. The cleavage products were fractionated by agarose gel electrophoresis and then transferred to a nylon membrane and processed for Southern blot hybridization. Another sample of the short fragments of infected-cell DNA from the sucrose gradient was 32 P labeled by random priming and used as a probe. The hybridization results revealed only three homologous bands in the *Bam*HI digest of the slowly sedimenting DNA (Fig. 4A, lane 1) and four bands in the *Eco*RI digests (Fig. 4A, lane 2). The bands that hybridized to the probe in the *Bam*HI digests corresponded to the virion DNA fragments *Bam*HI-K (8.7 kb) and *Bam*HI-R (6.1 kb), whereas the third predominant band proved not to be of viral origin (see below). Similarly, two of the four bands that hybridized to the short-fragment probe in the *Eco*RI digests corresponded to HCMV virion DNA fragments representing *Eco*RI-V (4.2 kb) and *Eco*RI-Q (5.8 kb). The same Southern blot that was used for the hybridization to the short-fragment DNA probe was then stripped of that probe and rehybridized to a mitochondrial DNA probe (Fig. 4A, lanes 5 to 8). The mitochondrial probe hybridized to the largest *Bam*HI fragment (16.8 kb; Fig. 4A, lane 6) and to two of the *Eco*RI fragments (9.0 and 8.2 kb; lane 8). The expected 1.2-kb *Eco*RI fragment of mitochondrial DNA was also present but ran off the gel in this experiment (data not shown). The viral band that gave the strongest autoradiographic image on the X-ray film was *Eco*RI-V, followed in order by *Bam*HI-K, *Eco*RI-Q, and *Bam*HI-R, which all lie adjacent to one another in a single block located near the middle of the

FIG. 3. Selective amplification of *Bam*HI-K compared with *Bam*HI-Q DNA sequences with increasing time and increasing doses of DHPG. (A) Total intracellular DNA prepared at different times after HCMV infection in the presence of either 1 or 25 µg of DHPG per ml was applied to filters and analyzed by dot blot hybridization with either a *Bam*HI-K or a *Bam*HI-Q plasmid DNA probe. The results are plotted as percentages of the level of hybridization to untreated infected cell DNA samples. (B) Total intracellular DNA prepared at 4 days after HCMV infection in the presence of different concentrations of DHPG was cleaved with either *Bam*HI or *Eco*RI and analyzed by Southern blot hybridization with a ³²P-labeled probe containing a 1:2 mixture of the *Bam*HI-K and *Bam*HI-Q plasmid DNAs.

unique L region in the physical map of the HCMV genome (Fig. 4B).

To confirm our interpretation that the amplified HCMV DNA sequences obtained in the presence of DHPG (referred to as DHPG DNA) contained predominantly *Bam*HI-K and *Eco*RI-V sequences, we isolated slowly sedimenting DNA fragments from the top of another sucrose gradient (pooled fractions 1 to 8) and used them as both the probe and the target for hybridization with selected cloned HCMV DNA fragments. A comparison between the DHPG DNA short fragments and intact total virion DNA after hybridization with plasmid DNA containing the *Bam*HI K fragment is shown in Fig. 5A. The single band obtained in the lane representing the DHPG DNA fragments (Fig. 5A, lane 1) matches the corresponding band in the virion DNA (lane 2). Similarly, the *Eco*RI digests of the DHPG DNA (lane 3) and the intact virion DNA (lane 4) show three comigrating bands representing the expected homology between *Bam*HI-K sequences and the *Eco*RI-V, *Eco*RI-Q, and *Eco*RI-E species. The same Southern blot was then stripped of the *Bam*HI-K probe and rehybridized to a *Bam*HI-Q probe. The *Bam*HI Q fragment, which is derived from the right-hand end of the viral genome, detected the appropriate fragments in both the *Bam*HI and *Eco*RI digests of virion DNA (lanes 6 and 8) but did not yield any signal with the small DHPG fragment DNA sample after digestion with either *Bam*HI or *Eco*RI (lanes 5 and 7).

The reciprocal experiment with cloned DNA from the *Bam*HI-K and *Bam*HI-Q plasmid DNA samples placed on the Southern blot was also carried out (Fig. 5B). Hybridization with the short DHPG DNA probe revealed the presence of *Bam*HI-K sequences only (lane 1) and not any *Bam*HI-Q sequences (lane 2). To confirm that the input *Bam*HI-Q plasmid DNA sequences were present on the blot in the appropriate ratios, the same Southern blot membrane used in lanes 1 and 2 was stripped and hybridized to a probe prepared by labeling total HCMV virion DNA. This control experiment showed that the lane representing the *Bam*HI Q fragment (lane 4) actually gave a more intense autoradiographic image than that of *Bam*HI-K (lane 3), suggesting that there was in fact more *Bam*HI-Q DNA than *Bam*HI-K DNA on the blot. Therefore, we conclude that there is selective amplification of sequences complementary to *Bam*HI-K in the presence of DHPG and that the *Bam*HI-Q fragment from the extreme right-hand end of the genome in the short unique segment is not represented in the DHPG-truncated small DNA fragments.

Since the *Hind*III M fragment from the extreme left-hand end of the long unique region was also totally absent from short-fragment DNA samples (Fig. 1C) and because hybridization experiments with the short DHPG fragment probes produced no evidence for the presence of any other viral sequences except for those within or adjacent to the *Bam*HI K and *Eco*RI V fragments, we also conclude that this procedure results in amplification of DNA from just a single localized domain within the HCMV genome. Logically, this site must represent a lytic origin of replication, which continually reinitiates in the presence of DHPG, despite a reduction in size of the replicating DNA molecules caused by the chain-termination effects induced by incorporation of DHPG near the end of the growing primer (35).

Direction of replication. The DHPG-induced chain-termination mapping approach also was used to study the direction of HCMV DNA replication. We reasoned that there should be a decreasing gradient of enrichment of the sequences on one or both sides of the origin and that larger

FIG. 4. Identification of the most highly amplified sequences. (A) Pooled DNA from fractions 3 to 8 in a sucrose gradient sedimentation profile of DHPG-treated intracellular DNA was analyzed by Southern blot analysis with ^{32}P -labeled probes representing either the small DNA fragments themselves (lanes 1 to 4) or platelet mitochondrial DNA (lanes 5 to 8). The size marker reference lanes contain *Bam*HI-digested (lane 1) or *Eco*RI-digested (lane 3), ^{32}P -labeled HCMV virion DNA, whereas lanes 5 and 7 contain unlabeled HCMV virion DNA. DNA fragments representing human mitochondrial DNA are indicated by the solid arrowheads (◄). (B) Physical maps of the relevant portion of the central U_L segment of the HCMV (Towne) genome showing relative positions, sizes, and overlaps of *Hind*III, *Bam*HI, and *Eco*RI restriction fragments and the location of the abundant *Eco*RI-V species found in the short DHPG-treated HCMV DNA molecules (open bar). Cloned fragments used as probes in Fig. 5, 6, and 7 are shown as solid bars. The nomenclature used and estimated fragment sizes in kilobases are derived from LaFemina and Hayward (26; unpublished data). Genomic sequence coordinates given for the *Hind*III fragments are those for the equivalent species in the HCMV (AD169) genome (4a).

DHPG fragments should contain more of the flanking sequences than smaller DHPG fragments. Fractions 1 to 4 (small fragments) and 5 to 8 (intermediate-sized fragments) from a sucrose gradient separation of DHPG DNA fragments were pooled; the DNA was digested with *Eco*RI and then electrophoresed in a 1% agarose gel and hybridized with a probe containing the *Hind*III D HCMV DNA fragment from the left-hand side of *Bam*HI-K (Fig. 6B). Because the fragments in the intact virion DNA lane must be present at equimolar concentrations, the intensity of each band should therefore be proportional to the size of the segment of the fragment that is complementary to the *Hind*III probe (Fig. 4B). Accordingly, the probe detected the corresponding bands in the intact virion DNA control digest (lane 6) in the following order of decreasing intensity: *Eco*RI-D = *Eco*RI-K $\gg$ *Eco*RI-Q. However, the lane representing the DNA from fractions 1 to 4 (lane 7) shows a faint *Eco*RI-Q band only, whereas all three fragments were displayed in the lane

FIG. 5. Confirmation that the slowly sedimenting DHPG DNA contains amplified HCMV *Bam*HI-K sequences. (A) Southern blot analysis of small fragment DNA from DHPG-treated cultures with cloned viral DNA probes. SF, *Bam*HI or *Eco*RI digests of pooled DNA from isolated small fragments obtained from fractions 1 to 8 of a sucrose gradient sedimentation profile of DHPG-treated DNA. V, *Bam*HI or *Eco*RI digests of cleaved unlabeled DNA extracted from HCMV virions. Lanes: 1 to 4, hybridization with ^{32}P -labeled *Bam*HI-K plasmid DNA probe; 5 to 8, ^{32}P -labeled *Bam*HI-Q probe. (B) Southern blot analysis with small DHPG DNA fragments as probes. Lanes: 1 and 3, *Bam*HI-cleaved *Bam*HI-K plasmid DNA; 2 and 4, *Bam*HI-cleaved *Bam*HI-Q plasmid DNA; 1 and 2, hybridization with ^{32}P -labeled small DHPG DNA fragment probe; 3 and 4, hybridization with control ^{32}P -labeled total virion DNA probe.

representing the DNA from fractions 5 to 8 (lane 9) with relative intensities that decreased in the following order: *Eco*RI-Q = *Eco*RI-K $\gg$ *Eco*RI-D. Thus, the reversed relative intensity of the bands in intermediate-sized DHPG DNA compared with that in virion DNA strongly suggests that *Eco*RI-Q is represented at higher molar concentration in all DHPG fragments than are *Eco*RI-K and *Eco*RI-D. This finding, in conjunction with the other data described above, strongly suggests that HCMV DNA replication starts in

FIG. 6. Detection of a gradient of amplified sequences adjacent to *Bam*HI-K in DHPG-treated intracellular DNA. Comparison by Southern blot analysis of slow- and intermediate-sedimenting fractions of intracellular DNA from cultures infected with HCMV in the presence of DHPG. All DNA samples on the blot were cleaved with *Eco*RI. (A) 32 P-labeled intracellular DHPG DNA probe (fractions 1 to 8); (B) cloned 32 P-labeled *Hind*III-D probe; (C) 32 P-labeled human platelet mitochondrial DNA probe. Lanes: V, unlabeled HCMV virion DNA; SF, small DNA fragments from sucrose gradient fractions 1 to 4; IF, intermediate-sized DNA fragments from sucrose gradient fractions 5 to 8; D, total intracellular DNA from DHPG-treated HCMV infected cells; M, total intracellular DNA from mock-infected cells. Bands corresponding to individual viral DNA fragments are identified by the standard nomenclature (see map in Fig. 4B), and mitochondrial DNA fragments are denoted by arrowheads ($\blacktriangle$).

*Bam*HI-K or *Eco*RI-V and proceeds leftward toward *Eco*RI-Q, then to *Eco*RI-K, and later to *Eco*RI-D.

The same Southern blot after hybridization to a probe made from labeled DNA prepared from fractions 1 to 8 (a mixture of both slow- and intermediate-sedimenting DHPG-DNA fragments) detected five major bands in the intact virion DNA control sample (Fig. 6A, lane 1). These bands correspond to *Eco*RI-V and *Eco*RI-E, in addition to the *Eco*RI-D, -K, and -Q species detected above. Since our results with the smallest DHPG fragments imply that the origin itself is located within *Bam*HI-K and *Eco*RI-V, the appearance of *Eco*RI-E (lane 4), which is just to the right of *Eco*RI-V on the physical map (Fig. 4B), suggests that the replication also proceeds to the right from *Eco*RI-V toward *Eco*RI-E, as well as to the left. This possibility is further supported by the appearance of *Eco*RI-V (and some *Eco*RI Q fragments) in the smallest DHPG DNA sample (fractions 1 to 4; lane 2) and the addition of *Eco*RI-E, -D, and -K in the intermediate-sized DNA from fractions 5 to 8 (lane 4). Again, some of the additional bands that were detected by the probe from fractions 1 to 8 in the intermediate-sized fragment position were found to be of mitochondrial origin (Fig. 6C, lane 14), and they correspond to the only two bands detected at this size position in mock-infected cells (lane 15).

For further analysis of the direction of replication, we used two probes in combination, one from just to the right and one from just to the left of the putative origin. Simultaneous hybridization with the *Hind*III-D (left of *Bam*HI-K) and *Bam*HI-C (right of *Bam*HI-K) probes gave a different relative intensity for *Eco*RI-E compared with *Eco*RI-D in the intermediate-sized DNA from fractions 5 to 8 (Fig. 7A, lane 4) compared with their relative intensity within the virion DNA sample (lane 1). This result suggests that *Eco*RI-E is present at a severalfold higher molar concentration than

*Eco*RI-D. Similarly, *Eco*RI-Q is more abundant than *Eco*RI-W, -K, or -D, and *Eco*RI-K is more abundant than *Eco*RI-D (Fig. 4B). The presence of a 5- to 10-fold overabundance of sequences complementary to *Bam*HI-K compared with *Bam*HI-Q in this same sample of intermediate-sized DHPG fragments was also confirmed by simultaneous hybridization with *Bam*HI-K and *Bam*HI-Q probes (Fig. 7A, lanes 6 to 10). The results from these experiments, when considered in conjunction with the other evidence described above, strongly suggest that the replication of HCMV DNA is bidirectional, proceeding both leftward and rightward from *Eco*RI-V.

For quantitation of this type of analysis, we studied the total intracellular viral DNA from DHPG-treated cultures by using dot blot hybridization, and we compared the levels of accumulation of sequences complementary to probes at the putative origin of replication relative to those adjacent to or distant from the origin (Fig. 7B). Again, there was greater amplification of sequences that are complementary to the *Bam*HI K fragment as compared with the sequences either to the left of the putative origin (*Hind*III-D) or to the right of the putative origin (*Bam*HI-C). Furthermore, the sequences complementary to the *Hind*III-D and *Bam*HI-C probes were also amplified severalfold relative to *Bam*HI-Q sequences from the short unique region at the extreme right-hand end of the genome. Therefore, this analysis (even with unfractionated DNA) again suggests that the origin of HCMV DNA replication lies within *Bam*HI-K, and that viral DNA replication proceeds both leftward toward *Hind*III-D and rightward toward *Bam*HI-C before reaching *Bam*HI-Q. A model illustrating the relative sizes and predicted map locations of the truncated replicated DNA fragments present in the DHPG-treated intracellular DNA pool is given in Fig. 7C. Note that we do not know whether individual DNA molecules proceed in both directions or whether the population is a mixture of some molecules proceeding leftward only and others proceeding rightward only. In addition, although they are depicted as linear structures, we cannot at present exclude the possibility of branched or circular structures.

DISCUSSION

The experiments described in this report represent a follow-up to our unexpected observation that HCMV DNA synthesis appeared to continue in the presence of DHPG even at inhibitor concentrations that resulted in complete cessation of infectious virion production (Hamzeh and Lietman, submitted). However, we now show that this result only applies to a specific segment of the viral genome, which becomes selectively amplified and progressively smaller in size. We imply that the amplified sequences result from continuous reinitiation on incompletely replicated viral DNA molecules that have become truncated by the incorporation of DHPG near the terminus of the growing primer strand, preventing further elongation (35). By measuring the relative abundance of viral DNA sequences in adjacent regions of the genome from sucrose gradient fractions containing DNA molecules of different sizes, we determined that the smallest molecules have the highest concentrations of origin sequences and that larger molecules contain increasing abundances of adjacent sequences that map to both the left and right of the origin sequences. Only a single origin locus appears to be present in the amplified DNA samples, and the sequences present at the highest concentration were found to map within the 4.2-kb *Eco*RI V and 8.5-kb *Bam*HI K fragments of HCMV (Towne). In fact, in the most highly

amplified samples these two species appear to be the only viral DNA fragments that remain in an intact form at all, although many DNA molecules are present that contain these sequences and are even smaller than the 4.2-kb *EcoRI*-V species. The *EcoRI* V fragment of HCMV (Towne) maps between nucleotide positions 88020 and 92210 within the unique L segment of the HCMV viral genome (map coordinates 0.367 through 0.388). These two fragments are equivalent to the *Bam*HI M and *Eco*RI V fragments of strain HCMV (AD169), which has been completely sequenced by Chee and colleagues in Cambridge (4a). Inspection of the DNA sequence of this region of the HCMV genome reveals that it includes the UL57 gene that gives rise to a 4.2-kb leftward transcript encoding the 140-kDa early single-stranded DNA-binding protein (DB140, equivalent to the UL29 or ICP8 major DNA-binding protein of HSV [1, 23]). However, upstream and to the right of UL57 there is a large 2.5-kb block of DNA sequences that has only small, poorly defined potential open reading frames (UL58 to UL60) and contains many interesting sequence elements and repetitive motifs. This region, which spans nucleotide positions 90340 through 92966, occupies the entire right-hand half of *Eco*RI-V and proceeds beyond it toward the right-hand boundary of *Bam*HI-K. An analysis of sequence features in this area, which we propose represents the lytic origin of HCMV, is illustrated in Fig. 8.

The HCMV DNA replication origin that we have identified here must function in the capacity of a lytic cycle origin that is uncoupled from cell cycle controls, but it lacks any recognizable DNA sequence or evolutionary homology with any previously identified viral DNA replication origin regions, including those in HSV and EBV. Therefore, the HCMV lytic origin represents a new fourth class of herpesvirus DNA replication origin that is distinctly different structurally from the HSV type of origin and from the EBV *ori*-P and *ori*-Lyt regions. Nevertheless, the genomic location adjacent to the 5' end of the gene for the single-stranded DNA-binding protein of HCMV parallels that of *ori*-L in HSV, and the entire 2.5-kb repetitive region may (like the HSV and EBV lytic origins) represent a noncoding intergenic domain. The presence of multiple ATF and core or

FIG. 7. Selective amplification of adjacent fragments on both sides of the putative lytic origin site. (A) Southern blot analysis of intermediate-sized DNA molecules from DHPG-treated intracellular DNA. Lane designations and arrangements as described in the legend to Fig. 6. All DNA samples on the blot were cleaved with *Eco*RI. Lanes: 1 to 5, mixed probe of *Bam*HI-C plus *Hind*III-D plasmid DNAs; 6 to 10, mixed probe of *Bam*HI-K plus *Bam*HI-Q plasmid DNAs. Relevant overlap maps are shown in Fig. 4B. (B) Quantitative dot blot hybridization analysis of the relative proportions of adjacent sequences derived from the right (*Bam*HI-C) or left (*Hind*III-D) of *Bam*HI-K in total intracellular DNA from HCMV-infected cells treated with DHPG at 10 μ g/ml. Again, the results are plotted as the percentage of radioactivity in the treated cultures at different times after infection compared with that in parallel untreated cultures. (C) Model for the derivation and structure of the small truncated DNA molecules containing *ori*-Lyt sequences found in intracellular HCMV DNA from DHPG-treated infected cells. In the smallest amplified molecules only the *Eco*RI V or *Bam*HI K fragments remain intact. Larger truncated molecules contain decreasing gradients of adjacent sequences on both sides of *Eco*RI-V. Therefore, a single predominant *ori*-Lyt site is proposed to lie within *Eco*RI-V. Although all of the slowly sedimenting DNA molecules are shown to be derived by bidirectional replication, we cannot at present exclude the possibility that some individual molecules proceed rightward only and others proceed leftward only.

FIG. 8. Structural organization of the predicted *ori-Lyt* locus. (A) Map location of the *ori-Lyt* locus (●) adjacent to the 5' end of the DB140 single-stranded DNA-binding protein gene (UL57, *dbp*, *ssb*) in the HCMV genome. The inverted repeats encompassing the unique L and S segments of the genome are shown as open bars. Major blocks of primordial herpesvirus genes that are evolutionarily conserved between EBV, HSV, and CMV are denoted by solid bars. *pol*, DNA polymerase; *hel*, helicase subunit. (B) Locations of repeated sequences and putative transcriptional control elements or factor binding sites within a 3.7-kb region on the right-hand side of the *Eco*RI V and *Bam*HI K fragments, i.e., HCMV (AD169) sequence coordinates 89700 to 93360. The coding region for the single-stranded DNA-binding protein (DB140) occupies the entire left-hand half of *Eco*RI-V. Symbols: ▽ (MLTF), consensus adenovirus major late transcription factor binding site (GGTCAGCTGACC); ◁ ▷, consensus TATAAA and AATAAA elements; ○, consensus CRE- or ATF-binding sites (TGACGTCA, TGACGACA, TGACGCA, TGACGTA, TGACGT); □, consensus SP-1 factor-binding sites (CCCGCCC); ◇, consensus CAAT transcription factor-binding site (TACCAAT); ◀▶, family of decamer dispersed repeat elements (at least 7 out of 10 matches with the consensus sequence AAAACACCGT); ●, family of seven *Fsp*I-*Sph*I 11-bp repeats (TGCGCATGCG); ▲, two 13-bp direct repeats (GAAAACCTATATA); ◆, five 19-bp direct repeats; GCCGGTAAAAAATTTTCCACT; ▼, two 28-bp direct repeats.

palindromic CRE sequences, plus a consensus MLTF site, is consistent with an association with transcriptional control elements and the presence of numerous other tandem and inverted repeat structures is typical of the local architecture within the DSL-DSR region encompassing the EBV lytic origin. Either of the repeated sequence elements GAAAA CCTATATA (two copies) or GCCGGTAAATTCCACT (five copies) within the tandemly repeated region might represent the anticipated A+T-rich initiation site(s). In addition, there are 23 interspersed copies of a decamer consensus element

AAAACACCGT between positions -250 and -1250 relative to the DB140 gene cap site (Table 1) and six clustered copies of the 11-bp TCGGCATGCGC *Fsp*I-*Sph*I element (Fig. 8), which both seem likely to be binding sites for either viral or cellular factors involved in regulation of transcription and DNA replication events.

The highly restricted host range and slow virus growth cycle, together with an unfortunate location of restriction enzyme cleavage sites, appears to have frustrated previous attempts to identify the HCMV origin by cotransfection plus

TABLE 1. Family of interspersed decamer repeat elements upstream from the promoters for the single-stranded DNA-binding protein genes in HCMV and SCM^a

Position and sequence of repeat element ^b			
HCMV (AD169)		SCMV (Colburn)	
-224 AAAATAGCGT	-233	-204 AAAATAGCGT	-213
-293 TAAACGACGT	-284	-254 AAAACAACGT	-263
-299 AGCACACCGT	-308	-296 AAAACAACGC	-287
-354 AAAACGCCAC	-363	-327 AAAACGGTGT	-336
-376 AAAACACCGT	-385	-339 AAAACACCGT	-330
-392 AAAACGGCGT	-401	-343 AAAACCAACGT	-352
-404 AAAACGCCGT	-395	-370 AAAACACCGC	-361
-455 AAAACACCGT	-464	-408 CAAACACCGT	-417
-474 CAAACACCGT	-465	-436 AAAGCAGCGT	-445
-498 AACACACCGT	-507	-454 AAAACACCGA	-445
-538 AAAACACCGT	-529	-474 AAAACGACGC	-465
-547 AAAGACCGC	-556	-573 AGAACACCGT	-564
-574 GAAACACCGC	-583	-593 AAAACGACGT	-602
-668 AAAACGCCGT	-677	-651 AAAACGGTGT	-660
-694 AAAACGCCGC	-703	-663 CAAACACCGT	-654
-754 CAAACGGCGT	-763	-679 AAAACACCGT	-688
-774 GAAACACGT	-765		
-906 AAAACACATT	-915		
-953 GAAACACCGT	-962		
-980 ACAACACCGT	-971		
-1047 AAAACACCGC	-1038		
-1096 AAACGCCGT	-1087		
-1139 AAAACGCCGT	-1130		
-1221 AACACACTGT	-1230		
-1232 AAACGCCGT	-1241		

^a Consensus sequences are AAAACACCGT for both HCMV and SCM^a. Deviations from the 10-bp consensus sequence are underlined.

^b Positions given are calculated relative to the consensus TATAAA-box elements (assigned to positions -28 to -23) in the HCMV (AD169) DB140 and SCM^a (Colburn) DB129 genes. Sequence data were obtained from Mark Chee (4a) and David Anders (in press).

superinfection procedures (R. LaFemina and G. S. Hayward, unpublished data; E. Mocarski, personal communication). However, in parallel studies, D. G. Anders and S. M. Punturieri (submitted for publication) have successfully used DNA transfection procedures with the Colburn strain of African green monkey simian CMV (SCMV) and defined a lytic cycle origin to within a 2.2-kb fragment located in a position that is exactly analogous to ours (i. e., just upstream from the promoter for the DB129 single-stranded DNA-binding protein gene [1]). DNA sequence analysis by those authors has also revealed 16 copies of the conserved interspersed decamer AAAACACCGT consensus element between positions -250 and -800 upstream from the DB129 gene mRNA start site in SCM^a DNA (Table 1), plus two copies of the 11-bp *FspI-SphI* element, together with multiple ATF and CRE elements.

As a confirmation of the general validity and complementarity of these two methods, we have also carried out chain termination plus hybridization with SCM^a (Colburn)-infected cells. Our results indicated that after 48 h of infection in the presence of DHPG there was selective amplification of the *EcoRI*-D, *Sall*-G and *Sall*-H, and *XbaI*-J, -M, and -U, -V, or -Y fragments. These DNA fragments define an exactly equivalent position at map unit 0.35 to 0.4 near the center of the unique L segment of the 220-kb SCM^a genome (29) and fulfill precisely the predictions from the DNA transfection-superinfection studies (Anders and Punturieri, submitted). In the reciprocal experiments with HCMV (Towne) DNA, Anders and Punturieri (submitted) obtained positive evidence for replication of the 22-kb *HindIII* A fragment but

found no activity with the *BamHI* K or C fragment, suggesting that a key element of the HCMV origin may be even farther than 2.5 kb upstream from the promoter for the major DNA-binding protein.

In viral genomes like those of HCMV and SCM^a that involve giant 220- to 240-kb replicons with relatively large origin domains, the chain-termination procedure appears to be a very useful complement to the DNA transfection approach. The transfection procedure cannot work with target DNA fragments that do not contain the entire origin domain in *cis* or if deletions or rearrangements of palindromic features occur within bacterial vector systems (as is the case with HSV *ori-L*). In contrast, the chain termination-hybridization procedure overcomes these obstacles by being applied directly within virus-infected cells and has the advantage of yielding information about directionality. However, the chain termination approach suffers at present by not permitting outside boundaries of the origin to be defined and therefore does not provide a great deal of precision about the exact location of key elements. Further potential development of this latter procedure for analysis of the structure of the replicating DNA fragments by electron microscopy and two-dimensional gel electrophoresis, etc., will undoubtedly be useful, although subject to the usual limitations of interpreting potentially aberrant effects caused by the use of inhibitors.

Despite plausible models for herpes simplex virus DNA replication occurring by a rolling-circle replication mechanism, that is not necessarily the case for CMV, and we do not consider it likely that the small HCMV DNA fragments accumulating in the presence of DHPG are produced by a rolling-circle mechanism. Although it has yet to be proved, the most likely scenario is that these fragments represent products resulting from multiple rounds of bidirectional initiation events that occur on shortened linear templates. Conceivably, there could be two populations with single growth forks proceeding in opposite directions, or individual molecules could contain multiple growth forks. It should be cautioned that, because they were generated in the presence of DHPG, the structure of the origin-enriched fragments does not necessarily make any predictions about the normal mode of HCMV replication. Nevertheless, it is interesting that tandem-repeat-defective CMV DNA of the type seen routinely in HSV has not yet been observed, despite deliberate attempts to generate it by passaging of SCM^a at high multiplicities of infection for over 20 successive passages (D. Ciuffo and G. S. Hayward, unpublished data). Instead, shortened, circularly permuted linear genome fragments of approximately 150 kb are rapidly accumulated during high-multiplicity passaging in HCMV infections (15, 24, 40). Furthermore, these defective DNA populations exhibit aberrant-sized forms of the *EcoRI* E and *BamHI* C fragments that could represent rearrangements across part of the *ori-Lyt* region (R. L. LaFemina, R. Pritchett, and G. S. Hayward, unpublished data).

The results described in this work should stimulate rapid future progress in identifying the proteins and specific *cis*-acting sequences involved in replication of the large and complex genomes of CMVs, and similar procedures have the potential for useful application to other herpesvirus systems as well.

ACKNOWLEDGMENTS

F.M.H. was supported by a scholarship from the University of Jordan, Amman, and by the Division of Clinical Pharmacology of the The Johns Hopkins University School of Medicine. The study

was supported by private grants to P.S.L. and by Public Health Service grant RO1-AI24576 to G.S.H. from the National Institutes of Health.

We thank Nadia Badiie and Mabel Chiu for excellent technical assistance, Pamela Wright for photography, and Sarah Heaggans for help with preparation of the manuscript. We are especially grateful to Mark Chee and Bart Barrell (Medical Research Council, Cambridge, England) for providing HCMV DNA sequence data and to David Anders (Wadsworth Center, Albany, N.Y.) for exchanging information about the *ori*-Lyt regions of HCMV and SCMV before publication.

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