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The Variable 3′ Ends of a Human Cytomegalovirus *ori*Lyt Transcript (SRT) Overlap an Essential, Conserved Replicator Element

LILI HUANG,[1] YUAO ZHU,[1] AND DAVID G. ANDERS[1,2]*

The David Axelrod Institute, Wadsworth Center for Laboratories and Research,[2] and Department of Biomedical Sciences, University at Albany School of Public Health,[1] Albany, New York 12201-2002

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The genetically defined human cytomegalovirus (HCMV) lytic-phase replicator, *ori*Lyt, comprises more than 2 kb in a structurally complex region that spans a variety of potential transcription control signals. Several transcripts originate within or cross *ori*Lyt, and we are studying these *ori*Lyt transcription units to determine whether they participate in initiating or regulating lytic-phase DNA synthesis. Results presented here establish the temporal accumulation and structure of the smallest replicator transcript, which we call SRT, and identify a single-sequence element essential to replicator function. SRT was detected as early as 2 h after HCMV infection of human fibroblast cells; transcript levels increased by 24 h and continued to increase thereafter. Consistent with its early appearance, treatment of HCMV-infected cells with the viral DNA polymerase inhibitor phosphonoformic acid had no effect on SRT accumulation; however, no SRT was detected in RNA preparations from cycloheximide-treated infected cells. Additional Northern (RNA) analysis localized the 0.2- to 0.25-kb SRT to an apparently noncoding segment near the center of the *ori*Lyt core region. Reverse transcriptase PCR (rapid amplification of cDNA 5′ ends [5′-RACE]) identified a single 5′ end. In transient-transfection assays, the sequence immediately upstream of SRT functioned as a promoter responsive to HCMV infection when placed upstream of a reporter gene, suggesting that SRT is the product of a discrete transcription unit. RNA ligase-mediated 3′-RACE showed that SRT is not polyadenylated and has heterogeneous 3′ ends within a roughly 45-nucleotide window overlapping an oligopyrimidine sequence having counterparts in the lytic-phase replicators of several herpesviruses. Mutation of the oligopyrimidine element showed that it is essential to *ori*Lyt replicator function; it is the only essential single-sequence HCMV *ori*Lyt replicator element described to date. Collectively, the location of SRT near the center of the *ori*Lyt core region, its early expression, its overlapping relationship with a sequence element essential to replicator function, and its similarities to replicator transcripts in other systems suggest the possibility that SRT plays a role in initiating or regulating HCMV lytic-phase DNA synthesis.

Human cytomegalovirus (HCMV), the prototype betaherpesvirus, is a clinically important cause of congenital viral infection and a frequent opportunistic pathogen in transplant recipients and AIDS patients (1, 30). HCMV contains a large, double-stranded, linear DNA genome of over 230 kbp (11, 12). The relative genomic complexity of HCMV is mirrored by its biology, as illustrated by the fact that the most relevant cellular reservoirs of latent virus genomes and sites of permissive replication have not been conclusively established, nor is HCMV pathogenesis well understood. Like other cytomegaloviruses (CMVs), HCMV has a highly species-specific host range but, within the permissive host, enters and apparently replicates in a wide variety of cell types (30, 58).

HCMV lytic-phase DNA replication has not been extensively studied, but the overall picture appears to resemble that of herpes simplex virus type 1 (HSV-1). In permissive cells, HCMV DNA is probably replicated by a rolling-circle mechanism that produces "endless" head-to-tail concatemers that are subsequently cleaved to unit length and packaged into preformed capsids (44, 48). DNA replication requires both virus-encoded proteins and a *cis*-acting replicator, *ori*Lyt. Eleven distinct loci are required for transient complementation of HCMV *ori*Lyt-mediated DNA replication (51, 52). Six of these loci encode herpesvirus group-common proteins that probably comprise the replication fork machinery, and four of the remaining five loci encode proteins that cooperate to upregulate expression of the replication fork proteins (36, 38). Whether any of the proteins encoded by the latter five loci are directly involved in initiating or performing DNA synthesis is not known. Despite the overall similarity to HSV-1 DNA synthesis, there are at least two significant differences between the genetic requirements for HCMV DNA replication and those for HSV-1. First, no HCMV homolog of the HSV-1 UL9 protein has been identified. The UL9 protein is required for HSV-1 DNA replication, binds specifically to several sites in each of the HSV-1 replicators, ori_S and ori_L, and is thought to employ its intrinsic DNA helicase activity for initial strand separation (10, 17, 18, 40, 41, 50, 63). Second, the HCMV replicator *ori*Lyt is large and structurally complex, whereas the HSV-1 replicators are comparatively simple (60, 61, 64). The molecular mechanisms underlying initiation of HCMV DNA replication are not known.

*ori*Lyt is the only HCMV lytic-phase replicator identified to date (2, 3, 28, 47). It spans roughly 2.4 kb within a structurally complex region near the middle of the long unique component, upstream of the UL57 open reading frame (ORF), and encompasses numerous potential transcription control signals, includ-

* Corresponding author. Mailing address: The David Axelrod Institute, Wadsworth Center for Laboratories and Research, P.O. Box 22002, Albany, NY 12201-2002. Phone: (518) 474-8969. Fax: (518) 474-3181. Electronic mail address: anders@wadsworth.org.

TABLE 1. Plasmids used in this study

Plasmid	Site/vector	Coordinates (nt)
pSP38Δ*Eco*RI	*Eco*RI-*Bam*HI/pGEM7Zf(−)	92210–95671
pLHB134	*Sma*I/pGL2-Basic	92500–92891
pLHB136	*Sma*I/pGL2-Basic	92500–93197
pLHB135	*Sma*I/pGL2-Basic	92636–92891
pLHB138	*Sma*I-*Xho*I/pGL2-Basic	92636–93197
pLHB137	*Sma*I-*Bgl*II/pGL2-Basic	92887–93197
pLHB135Δ2	*Sma*I/pGL2-Basic	92636–92645
pLHB135Δ8	*Sma*I/pGL2-Basic	92636–92711
pLHB135Δ9	*Sma*I/pGL2-Basic	92636–92749
pLHB135Δ11	*Sma*I/pGL2-Basic	92636–92785
pLHB135Δ3	*Sma*I/pGL2-Basic	92636–92823
pLHB135Δ24	*Sma*I/pGL2-Basic	92636–92861
pYZ9	*Nsi*I-*Xcm*I/pSP54	90504–94860[a]
pYZ9R2	*Nsi*I-*Xcm*I/pSP54	90504–94860[b]
pYZ9R	*Nsi*I-*Xcm*I/pSP54	90504–94860[c]

[a] The 22-nt sequence from nt 92471 to 92492 was replaced with a 6-nt *Pst*I site.
[b] The 22-nt sequence from nt 92471 to 92492 was replaced with a 22-nt random sequence.
[c] The 22-nt sequence from nt 92471 to 92492 was replaced with a 26-nt random sequence.

ing consensus transcription factor recognition sequences and polyadenylation signals. Preliminary Northern (RNA) analysis detected transcripts within *ori*Lyt. Transcriptional control regions and/or transcripts have been shown to participate in replicator function in many systems (16, 42), including other herpesviruses (27, 53, 55, 56, 65, 66). Therefore, we are studying the *ori*Lyt transcription units toward an understanding of their relationships to replicator function, if any. Here, we describe results characterizing the structure and expression of the smallest of these replicator transcripts and identifying an essential replicator sequence situated at the 3′ end of the transcribed region and discuss possible roles of this RNA in initiation of HCMV lytic-phase DNA synthesis suggested by this juxtaposition.

MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblast (HFF) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum. HCMV strain AD169 (American Type Culture Collection, VR-538) was passaged at a low multiplicity of infection in HFF cells and maintained as frozen, titered stocks. For RNA studies, HFF cells were infected at an approximate multiplicity of infection of 10 PFU per cell.

Plasmids. Recombinant plasmids made for this study are detailed in Table 1, and oligonucleotides used in their construction are listed in Table 2. Plasmids SP38, SP50, and SP54 have been described elsewhere (2). pSP38Δ*Eco*RI was constructed by removing from pSP38 the *Eco*RI fragment extending from the vector *Eco*RI site to the insert *Eco*RI site at nucleotide (nt) 92210. Luciferase reporter plasmids LHB134, LHB135, and LHB136 were made by excising from pSP50 the DNA fragments detailed in Table 1, blunting the cohesive ends by Klenow polymerase treatment, and inserting the respective fragments into the *Sma*I site of the luciferase reporter vector pGL2-Basic (Promega Corp., Madison, Wis.). pLHB137 was produced by removal of the *Bgl*II-*Not*I fragment extending from nt 92500 to 92887 from pLHB136, treatment with Klenow polymerase to blunt the noncomplementary cohesive ends, and religation. pLHB138 was constructed by removal of the *Xho*I fragment extending from nt 92500 to 92636 from pLHB136 and religation. A series of deletion mutants was generated from pLHB135 by partial digestion with *Sph*I: after partial cleavage of pLHB135 with *Sph*I, linearized plasmids were digested to completion with *Bst*Z1 and gel purified, then treated with Klenow polymerase to make blunt ends, gel purified again, and religated. pYZ9 was made by replacing the pSP54 *Nsi*I-to-*Xcm*I fragment with a mutated version of the same fragment that was made by using an overlap-extension PCR method (31). The mutated *Nsi*I-to-*Xcm*I fragment was produced by using YUAO19 and YUAO20 as overlapping central primers, R92206 and 92654 as flanking primers, and pSP54 as a template. The overlapping mutagenic primers introduced a unique *Pst*I site. pYZ9R and pYZ9R2 were made by ligating a linker composed of annealed oligonucleotides RP1 and RP2 or 9RP1 and 9RP2, respectively, into the pYZ9 *Pst*I site. All plasmids were checked by sequencing.

RNA isolation and Northern analysis. Total cellular RNA was prepared from uninfected and HCMV-infected HFF cells by a guanidinium-cesium chloride method (14, 24). Where indicated, the polyadenylated RNA fraction was isolated by oligo(dT)-cellulose chromatography (7). For some experiments, total cellular RNA was isolated at 96 h postinfection (p.i.) from cells treated with phosphonoformic acid (PFA) (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 200 μg/ml. Late RNA was isolated at 96 h p.i. from untreated infected cells. For time course studies, total cellular RNA was prepared from HCMV-infected cells at 2, 8, 12, 24, 48, 72, and 96 h p.i.

For Northern analysis, 2 μg of oligo(dT)-selected RNA, or 50 μg of total RNA (for temporal analysis), was fractionated by electrophoresis through a formaldehyde-containing 1% agarose gel (6) and then transferred to a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Hercules, Calif.). After transfer, the membrane was prehybridized for at least 2 h at 65°C in hybridization solution containing 50% (vol/vol) formamide, 0.12 M Na_2HPO_4 (pH 7.2), 0.25 M NaCl, 7% (wt/vol) sodium dodecyl sulfate (SDS), 500 μg of salmon sperm DNA per ml, and 500 μg of *Saccharomyces cerevisiae* tRNA per ml. Hybridization with an [α-^{32}P]CTP-labeled riboprobe was done at 65°C overnight in hybridization solution. For probe preparation, pSP38Δ*Eco*RI was linearized with either *Nsi*I (the 3′ overhang generated by *Nsi*I was blunted by treatment with Klenow polymerase) or *Sma*I and transcribed in vitro with T7 RNA polymerase by using the MAXIscript in vitro transcription system (Ambion, Austin, Tex.). After hybridization, the membranes were washed sequentially with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS, 0.5× SSC plus 0.1% SDS, and 0.1× SSC plus 0.1% SDS, at 67°C for 30 min per wash. Annealed probe was detected by exposing the washed membrane to Kodak X-AR film at −80°C with an intensifying screen.

5′-RACE. Rapid amplification of cDNA 5′ ends (5′-RACE) (8, 22) was performed with the Clontech 5′-RACE system (Clontech, Palo Alto, Calif.). Briefly, 2 μg of oligo(dT)-selected, infected-cell RNA isolated at 96 h p.i. was primed for reverse transcription with a gene-specific primer (either P19, P21, or P31 [Table 2]). Reverse transcription reactions were done at 52°C for 30 min, using avian

TABLE 2. Synthetic oligonucleotides used in 5′-RACE and RLM-3′-RACE

Oligonucleotide	Sequence	Coordinates (nt)
P19	5′-AACGAAACGTTCTACGAAAACGGAC-3′	92423–92447
P20	5′-TGGCGGAACGGGAACCACCGTAACC-3′	92448–92472
P21	5′-GGGGGCAAATTTTTACCAAATTTGGGC-3′	92511–92537
P22	5′-CCATGATTTCCAATGGGACGGCGTTTC-3′	92540–92566
P23	5′-GAAACGCCGTCCCATTGGAAATCATGG-3′	92566–92540
P24	5′-ATAATCCGCGTGGCAACGCCCTGACAA-3′	92625–92599
P31	5′-CGTCATCCTGTGGAATTCCGGACATAC-3′	92198–92224
YUAO19	5′-CGGAGGAGAACTGCAGTTACGGTGGTTCCCGTT-3′	92502–92454[a]
YUAO20	5′-ACCACCGTAACTGCAGTTCTCCTCCGGAACCGG-3′	92461–92509[a]
R92206	5′-GACTATGCATGTGGAATTCCGGACATACG-3′	92206–92225
92654	5′-CGGCGCATGCGCACTCGAGT-3′	92654–92634
RP1	5′-TTCTAGAACCGCTGGATGCA-3′	
RP2	5′-TCCAGCGGTTCTAGAATGCA-3′	
9RP1	5′-TTCTAGACGCGATGCA-3′	
9RP2	5′-TCGCGTCTAGAATGCA-3′	

[a] The sequence between nt 92492 and 92471 in primers YUAO19 and YUAO20 was replaced with a 6-nt *Pst*I site.

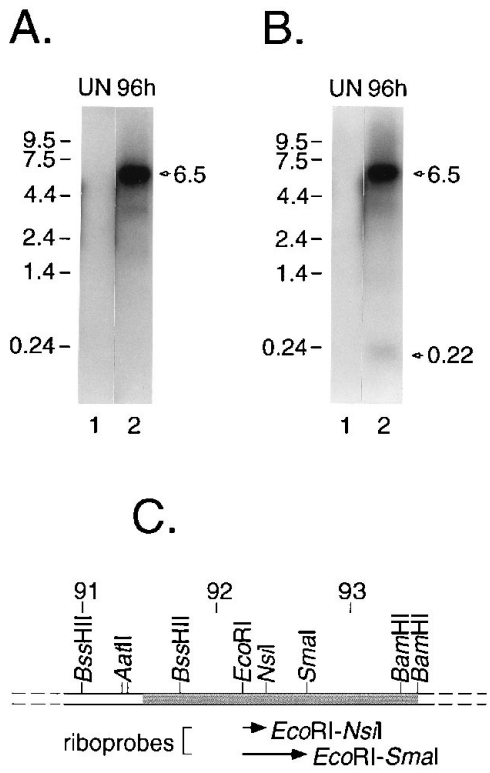

FIG. 1. Northern analysis localizing SRT. Northern transfers of oligo(dT)-selected RNA (2 μg) prepared from uninfected cells (UN) or HCMV-infected cells at 96 h p.i. (96h) were hybridized with the indicated ^{32}P-labeled riboprobe as detailed in Materials and Methods. Bound probe was detected by autoradiography. Postions of molecular size markers (0.24- to 9.5-kb RNA ladder; Gibco BRL) and estimated sizes of detected RNAs (in kilobases) are noted to the left and right of the gels, respectively. (A) Hybridization with the 188-bp *Eco*RI-*Nsi*I riboprobe. (B) Hybridization with the 495-bp *Eco*RI-*Sma*I riboprobe. (C) Riboprobes used for experiments depicted in panels A and B. The core region of *ori*Lyt is grayed.

myeloblastosis virus reverse transcriptase (RT). Negative-control reactions were carried out in parallel by omitting the RT. RNA then was hydrolyzed with NaOH, and cDNA was purified by being bound to silica matrix in a high salt concentration. The 3′ end of the purified cDNA was ligated to the supplied anchor oligonucleotide (5′-P-CACGAATTCACTATCGATTCTGGAACCTTC AGAGG-NH_2-3′) with bacteriophage T4 RNA ligase. One percent of the ligated cDNA was amplified by using *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) in a 50-μl reaction mixture including 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl; pH 8.3), 2 mM $MgCl_2$, 0.2 mM each of the four deoxynucleoside triphosphates, the supplied anchor primer (5′-CTGGTTCGGCCCACCTC TGAAGGTTCCAGAATCGATAG-3′), and the indicated nested gene-specific primer (Table 2). For maximum amplification specificity, hot-start PCR was done. After all of the reaction components except the primers were briefly heated to 82°C, the primers were added and then the following thermocycler sequence was started: an initial denaturation at 94°C for 3 min; then 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min; and a final extension at 72°C for 7 min. PCR products were separated by electrophoresis through a 2% agarose gel and visualized with ethidium bromide. Detected bands were gel purified and ligated into a TA cloning vector, pCR (Invitrogen Corp, San Diego, Calif.). Cloned inserts were sequenced by the dideoxynucleotide method with Sequenase (U.S. Biochemicals, Cleveland, Ohio) and M13 forward (5′-GTTTTCCCAGTC ACGAC-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) primers or with an Applied Biosystems automated sequencer. cDNA sequences were aligned with the published HCMV strain AD169 sequence (accession no. X17403) (12) using the Genetics Computer Group sequence analysis package (23).

RLM-3′-RACE. For RNA ligase-mediated rapid amplification of cDNA 3′ ends (RLM-3′-RACE) (45), 1 μg of oligo(dT)-selected, HCMV-infected-cell RNA was mixed with 16 pmol of the anchor oligonucleotide with its 5′ end phosphorylated and its 3′ end blocked by an amino group, and the mixture was heated to 90°C for 2 min. The 5′ end of the anchor oligonucleotide then was ligated to RNA 3′ ends by using 60 U of T4 RNA ligase in the presence of 60 U of RNase inhibitor at 16°C overnight. Unligated anchor oligonucleotide was removed by centrifugal ultrafiltration using Centricon 30 microconcentrators (Millipore Corp., Bedford, Mass.), with several washes of diethyl pyrocarbonate-treated water. Anchor primer then was used to prime first-strand cDNA synthesis by SuperScript RT (Gibco BRL, Gaithersburg, Md.). Reverse transcription was carried out at 42°C for 30 min, and the RNA strand then was degraded by addition of RNase H (Gibco BRL). One-tenth of the cDNA was amplified by using the anchor primer and a gene-specific primer, P24 (Table 2). For further amplification of the signal, a second round of PCR was performed using the anchor primer and either P24 or the nested gene-specific primer P23 (Table 2). PCR, cloning, and sequencing procedures were as described for 5′-RACE.

Transfection and luciferase assay. LipofectAMINE reagent (Gibco BRL) was used to transfect plasmid DNAs into HFF cells as described previously (29, 36). Briefly, HFF cells were plated onto six-well plates, at a cell density of 3.5×10^5 per well, 24 h prior to DNA transfection. One microgram of luciferase reporter construct was transfected per well. The cells were infected with HCMV at a multiplicity of infection of 5 to 10 PFU per cell 24 h after transfection, and then harvested 24 or 96 h after infection for luciferase assay, which was carried out with the Promega luciferase assay system. Luciferase activity was measured by using a scintillation counter with the coincidence feature turned off. The linear range of light detection was determined by using purified firefly luciferase (Boehringer Mannheim Corp., Indianapolis, Ind.). All transfections were done in triplicate, at least twice.

Transient *ori*Lyt replication assay. HFF cells were plated onto 6-cm-diameter dishes at a cell density of 5×10^5 per dish and 24 h later were transfected with 10 μg of plasmid DNA per dish by a calcium phosphate method, as described previously (13, 52). The transfected cells were infected with HCMV at an approximate MOI of 10 PFU per cell 24 h after transfection. After 96 h of infection, total cellular DNA was isolated, treated with *Dpn*I plus *Eco*RI, subjected to electrophoresis through a 0.8% agarose gel, transferred to a Zeta-Probe nylon membrane, and probed with random-primer ^{32}P-labeled pGEM7Zf(−).

RESULTS

Identification and temporal expression of *ori*Lyt RNA. Preliminary Northern analysis detected at least four transcripts from within and around *ori*Lyt, all extending from right to left in the standard arrangement of the virus genome (32, 33). The two largest of these originate to the left of the *ori*Lyt core region, upstream of the UL57 ORF, which they cross. A moderately abundant late RNA of about 400 to 700 nt spans the UL59 ORF, which lies at the leftward boundary of the *ori*Lyt core region, and an abundant 6.5-kb transcript originates well to the right of the core *ori*Lyt region, extends through *ori*Lyt, and coterminates with the UL59 transcripts, which use the polyadenylation signal (AAUAAA) at nt 91184 (33). Finally, we observed a second small RNA of about 220 nt that was detected only by probes from within the core region of *ori*Lyt, suggesting that it both initiates and terminates within *ori*Lyt. To better localize this smallest RNA, we performed Northern analysis using oligo(dT)-selected RNA harvested at 96 h p.i. and several strand-specific riboprobes (Fig. 1). An *Eco*RI-to-*Nsi*I riboprobe (nt 92210 to 92398) detected only the 6.5-kb transcript (Fig. 1A, lane 2). In contrast, an *Eco*RI-to-*Sma*I riboprobe (nt 92210 to 92705) detected both the 6.5-kb transcript and the smaller 0.22-kb RNA (Fig. 1B, lane 2). Neither probe detected specific transcripts in RNA preparations from uninfected cells (Fig. 1A and B, lanes 1), nor did opposite-strand probes detect specific signals (32). Riboprobes extending rightward from the *Sma*I site detected the 6.5-kb transcript but not the 0.22-kb RNA (data not shown). We concluded from these results that the 0.22-kb RNA must be complementary to a DNA sequence between the *Nsi*I site at nt 92398 and the *Sma*I site at nt 92705.

The temporal accumulation of the 6.5- and 0.22-kb RNA species was examined by Northern analysis of total cell RNA preparations from HCMV-infected cells harvested at 2, 4, 8, 12, 24, 48, 72, and 96 h p.i., using the *Eco*RI-to-*Sma*I riboprobe. To determine whether these transcripts are made in the absence of DNA replication, we also examined RNA harvested at 96 h p.i. from HCMV-infected cells maintained in 200 μg of PFA per ml of medium (Fig. 2). The 6.5-kb transcript was first

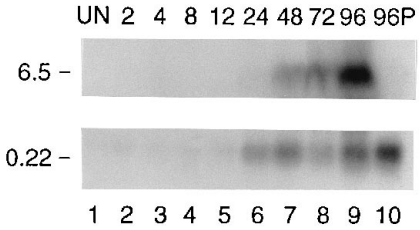

FIG. 2. Temporal accumulation of the 6.5- and 0.22-kb transcripts. Total cellular RNA (50 μg) prepared from uninfected cells (UN) (lane 1), HCMV-infected cells harvested at various times p.i. (as indicated above the lanes) (lanes 2 to 9), or from infected cells treated with 200 μg of PFA per ml medium harvested at 96 h p.i. (96P) (lane 10) was examined on a Northern blot using the ^{32}P-labeled 495-nt *Eco*RI-*Sma*I riboprobe. Bound probe was detected by autoradiography. Only the respective portions of the autoradiogram including the indicated transcripts are shown. Estimated sizes of transcripts are given in kilobases on the left.

detected at 24 h p.i., increased dramatically at 48 h p.i., and continued to increase through 96 h p.i. (Fig. 2, lanes 6 to 9). In RNA prepared from cells infected in the presence of PFA, the 6.5-kb transcript was barely detectable (Fig. 2, lane 10). Taken together, these data suggest that the 6.5-kb transcript is a leaky-late (γ1) RNA. In contrast, the 0.22-kb RNA was detected as early as 2 h p.i., was present at low levels through 12 h p.i., increased in abundance by 24 h p.i., and continued to accumulate at late times (Fig. 2, lanes 2 to 9). Also unlike the 6.5-kb RNA, PFA treatment did not inhibit accumulation of the 0.22-kb RNA (Fig. 2, lane 10). Neither the 6.5-kb nor the 0.22-kb RNA was detected in RNA preparations from cycloheximide-treated, infected cells harvested at 24 h p.i. in which the major immediate-early transcripts were readily detected (data not shown).

Mapping the 5′ end of the small replicator transcript using 5′-RACE. Because its temporal expression and its location near the center of the HCMV *ori*Lyt core region are consistent with the possibility of a role in the replicator function of *ori*Lyt, we used PCR-based approaches to establish the structure of the 0.22-kb RNA, which we called the small replicator transcript (SRT). On the basis of the Northern analysis results that placed SRT between the *Nsi*I and *Sma*I sites, we designed several sequence-specific primers for 5′-RACE; the positions of these primers are depicted in Fig. 4A, and their sequences given in Table 2. Gene-specific primer P19 or P21 was used to synthesize a first-strand cDNA from oligo(dT)-selected RNA prepared from cells at 96 h p.i. After their 3′ ends were ligated to an anchor oligonucleotide, the cDNAs were amplified by using the complementary anchor primer and a nested, sequence-specific primer. Either primer P20 or P22 was used to amplify P19-synthesized cDNA, and P22 was used to amplify P21-synthesized cDNA (Fig. 3). Two negative controls also were done. First, RT-minus cDNA reaction mixtures were amplified in parallel to test for contamination of RNA preparations with genomic DNA. Second, equivalent PCRs were done without cDNA to test for possible oligonucleotide polymerization. PCR products were separated by electrophoresis through a 2% agarose gel. Figure 3 shows gel photographs of two independent, representative experiments. A distinct band of 190 bp was seen after P21-synthesized cDNA was amplified with primer P22 (Fig. 3A, lane 1, and B, lane 2). No specific band was noted after amplification of P19-synthesized cDNA using the P20-anchor primer pair (Fig. 3A, lane 3), but a faint 190-bp band was detected after amplification of P19-synthesized cDNA with primer P22 and anchor primer (Fig. 3B, lane 3). No distinct bands were detected in the negative controls (e.g., Fig. 3A, lanes 2 and 4, and B, lane 1). In another experiment, we used the sequence-specific primer P31 to synthesize cDNA. The P31 sequence lies in a downstream segment, outside the SRT region defined by Northern analysis (Fig. 4A). The resulting cDNA was amplified with the anchor primer and P22; consistent with the Northern blot results, no distinct bands were produced (Fig. 3B, lane 4). The 190-bp DNA fragments from positive amplifications were subsequently gel purified, cloned, and sequenced (Fig. 4). Seven molecularly distinct clones revealed 5′ ends within a 4-nt window around nt 92685: two showed a 5′ end at nt 92683, two showed a 5′ end at nt 92685, and three showed a 5′ end at nt 92686 (Fig. 4A). The limited 5′-end heterogeneity that was observed in the cDNA clones could be due either to slight variation in start sites or to incomplete cDNA synthesis. On this basis, we designated nt 92686 the 5′ end of SRT.

Mapping the 3′ end of SRT by RLM-3′-RACE. Conventional 3′-RACE, in which an oligo(dT)-adapter primer is used to synthesize a first-strand cDNA and the cDNA is then amplified with an adapter primer and a gene-specific primer, failed to produce specific DNA products in repeated attempts with two different primer sets. In parallel experiments, this standard 3′-RACE procedure readily amplified the 3′ ends of the UL59 transcripts (33). These results, together with the absence of an appropriately positioned polyadenylation signal, suggested that SRT might not be polyadenylated even though it was enriched in oligo(dT)-selected RNA preparations. Therefore, as an alternate approach, RLM-3′-RACE was attempted. After ligation of an anchor sequence to RNA 3′ ends, first-strand cDNAs were synthesized by using a complementary anchor primer and then amplified by using the anchor primer and a

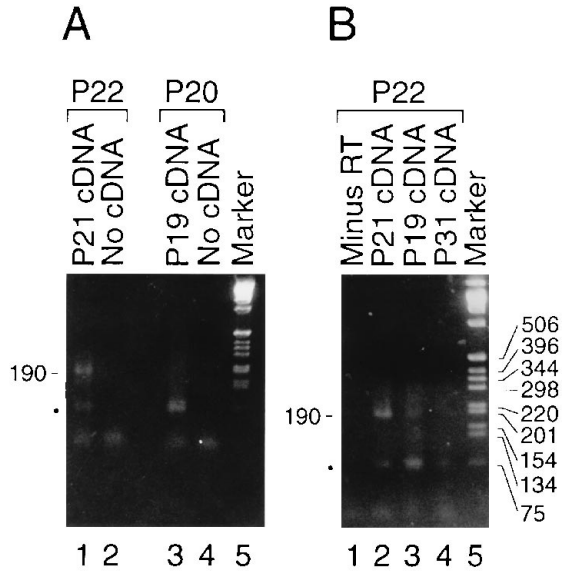

FIG. 3. Mapping of the 5′ end of the 0.22-kb RNA. Photographs of agarose gels from two independent 5′-RACE experiments which were done as described in Materials and Methods are shown. P19, P21, and P31 cDNAs are first-strand cDNAs synthesized from oligo(dT)-selected RNA using primers P19, P21, and P31, respectively. (A) Lane 1, P21-synthesized cDNA amplified by using the anchor primer plus a nested primer P22; lane 3, P19-synthesized cDNA amplified by using the anchor primer plus a nested primer P20; lanes 2 and 4, no cDNA amplification with anchor primer plus the indicated nested primers. (B) Lane 1, control cDNA synthesis without RT; lanes 2 to 4, P19-, P21-, and P31-synthesized cDNAs, respectively, individually amplified by using the anchor primer plus primer P22. Size markers (1-kb DNA ladder; BRL) (lanes 5) are noted in base pairs on the right. The detected 190-bp band is noted on the left. A roughly 75-bp primer-dimer band that appeared in some reactions is indicated (dot).

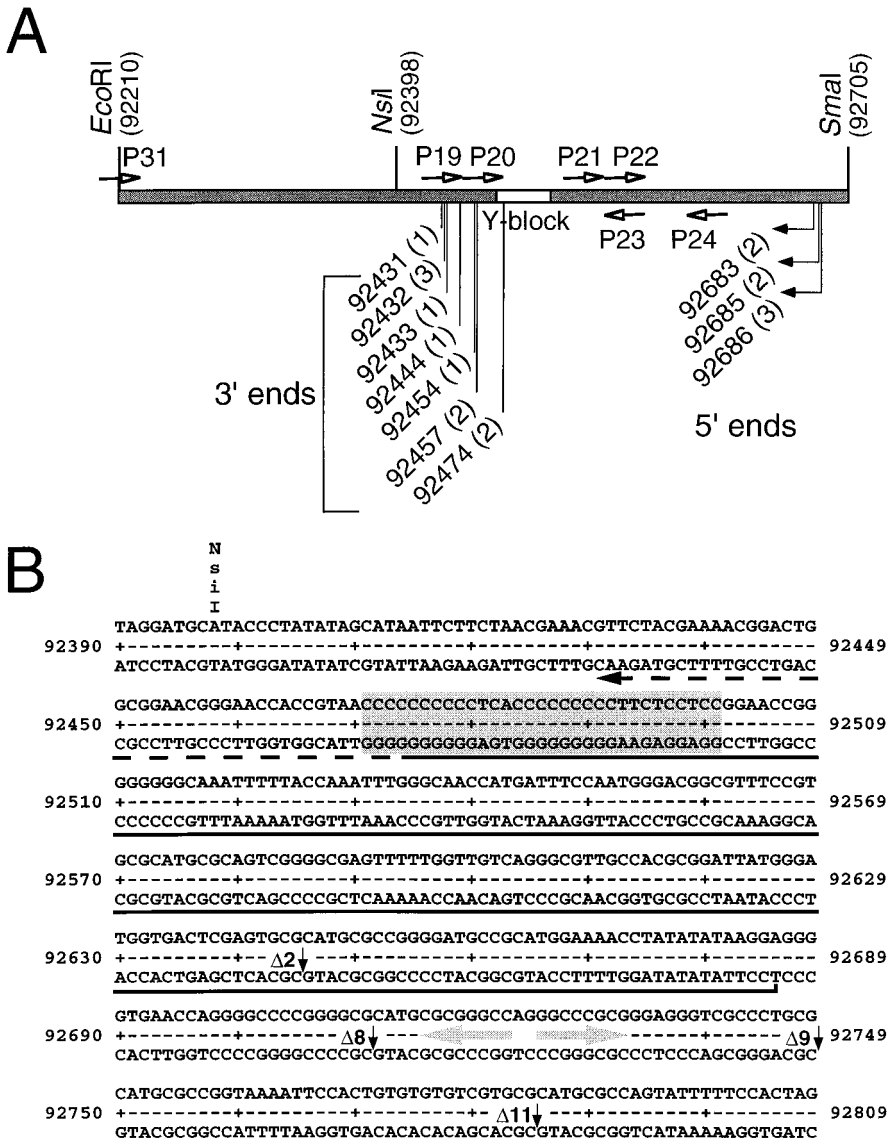

FIG. 4. Summary of SRT mapping results. (A) Distribution of SRT termini determined by cloning and sequencing of 5′-RACE and RLM-3′-RACE products. In parentheses are the number of sequenced clones (1 to 3) terminating at each position. The primers used in 5′-RACE and RLM-3′-RACE (arrows) and the Y-block (open box) are indicated. (B) Sequence of SRT and flanking regions. SRT (black arrow below the lower strand; the dashed line represents the heterogeneous 3′ ends), the Y-block (gray box), and a dyad symmetry 5′ of SRT (gray arrows) are indicated. Also noted are the upstream boundaries of some of the deletions (Δ2, Δ8, Δ9, and Δ11) of the reporter construct pLHB135. HCMV (AD169) sequence data are from Chee et al. (12) (GenBank accession no. X17403).

gene-specific primer, P24. To further amplify the signal, a second round of PCR was done using the anchor primer and either P24 or a nested primer P23 (Fig. 5). P24 produced a broad band ranging from roughly 220 to 300 bp which included the anchor sequence (Fig. 5, lane 1), whereas P23 produced a broad band ranging from about 140 to 180 bp (Fig. 5, lane 2). The products of both P23 and P24 reactions were gel isolated and cloned, and the cloned fragments were sequenced. All of the sequenced clones derived from the nested primer P23 were specific to the small *ori*Lyt transcript region, whereas reamplification with P24 produced both HCMV-specific and nonspecific sequences. The 11 molecularly distinct, HCMV-specific clones that were sequenced had heterogeneous 3′ ends within a roughly 45-nt window extending from nt 92431 to 92474, overlapping an oligopyrimidine stretch (termed the Y-block; summarized in Fig. 4A). None of the sequenced clones included a poly(A) tail at the 3′ end, consistent with the absence of a polyadenylation signal (AAUAAA) and with the failure of conventional 3′-RACE. In contrast, UL59 transcripts that were cloned by the RLM-3′-RACE method all showed polyadenylation (32, 33). Together with the 5′-RACE data, these results define SRT as an unspliced, nonpolyadenylated RNA of 210 to 255 nt with a precise 5′ end and heterogeneous 3′ ends over a roughly 45-nt window that overlaps the Y-block (Fig. 4B).

A candidate promoter upstream of SRT. The small, nonpolyadenylated SRT could be made either directly, as the product of a discrete transcription unit, or through processing of a larger precursor. Synthesis from a discrete transcription unit would require that a cognate promoter be situated upstream of, or within, the SRT region. Therefore, to search for a candidate promoter we made two pairs of luciferase reporter

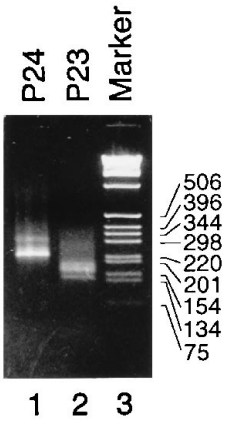

FIG. 5. Mapping of the 3′ end of the 0.22-kb RNA. RLM-3′-RACE was carried out as described in Materials and Methods. Shown are the products of a representative second-round PCR separated by electrophoresis through a 2% agarose gel. Products of the initial P24-anchor primer PCR were reamplified by using either the P24-anchor primer pair (lane 1) or the P23-anchor primer pair (lane 2). Size markers (the 1-kb DNA ladder; BRL) (lane 3) are noted in base pairs on the right.

constructs and tested them in transient-transfection assays (Fig. 6). One pair extended downstream of the SRT 5′ end to position +185 and thus included almost the entire SRT region excluding the Y-block (pLHB134 and pLHB136), and the other pair extended downstream only to position +49 (pLHB135 and pLHB138). For these experiments, HFF cells were transfected with the indicated reporter plasmid and infected with HCMV 24 h later. Luciferase activity was measured at 24 and 96 h after infection. By 24 h p.i., cells transfected with pLHB134 (nt +185 to −206 relative to the defined start site) expressed about 7-fold-higher luciferase activity, and cells transfected with pLHB136 (nt +185 to −512) produced 20-fold-higher activity, than the promoterless vector pGL2-Basic. Likewise, pLHB135 (nt +49 to −206) produced about a 10-fold-higher activity, and pLHB138 (nt +49 to −512) produced about a 32-fold-higher activity, than pGL2-Basic. Minimal luciferase activity was found in equivalently transfected, uninfected cells. For each construct, the measured luciferase activity was 10- to 30-fold higher at 96 h p.i. than at 24 h p.i., but the relative luciferase activity produced by these plasmids with respect to each other was similar to that observed at 24 h p.i. The sequence between nt +49 and +185 made no significant contribution to luciferase expression (Fig. 6; compare pLHB134 with pLHB135 and pLHB136 with pLHB138). However, including the upstream segment extending from positions −206 to −512 produced a roughly threefold increase in activity relative to the plasmids extending only to nt −206 (Fig. 6; compare pLHB134 with pLHB136 and pLHB135 with pLHB138). pLHB137, which includes only the upstream segment (nt −202 to −512), also produced greater expression relative to that of pGL2-Basic. Overall, the strength of the candidate SRT promoter was comparable to that of the UL57 promoter in this assay (32, 36).

The findings that the sequence from nt +49 to −206 efficiently directed transcription across a linked reporter gene in transient-transfection assays and that this activity was strongly activated by HCMV infection were consistent with the presence of a functional promoter. To further characterize this candidate promoter region, we made a series of nested deletions in pLHB135 and measured their respective promoter activities, relative to that of pLHB135, in transient-transfection assays (Fig. 7). Again, transfections assayed at either 24 or at 96 h p.i. produced similar relative activities. Transfection with either pLHB135Δ24 (nt +49 to −176), pLHB135Δ3 (nt +49 to −138), or pLHB135Δ11 (nt +49 to −100) produced roughly 40 to 50% of the luciferase activity of the parent construct pLHB135; we found no statistically significant differences in activity between these plasmids. However, pLHB135Δ9 (nt +49 to −64) showed a further two- to threefold reduction of luciferase expression (to 10 to 15% of that of pLHB135), and luciferase expression of pLHB135Δ8 (nt +49 to −26) was indistinguishable from that of the promoterless pGL2-Basic at 24 h p.i. and was very low at 96 h p.i. Note that in the construction of pLHB135Δ8, a novel Sp1 consensus sequence was adventitiously formed at the distal junction of HCMV and vector sequences, which could contribute to residual activity at late times. Taken together, these results suggest that the sequence between nt +49 and −100 relative to the SRT 5′ end may span a functional promoter and that additional flanking sequences may augment this promoter activity.

Evidence that the Y-block is essential to replicator function. The finding that the variable 3′ ends of SRT lay within or directly downstream of the Y-block sequence was of interest because similar elements are present in the lytic replicators of simian and murine CMVs, as well as the lytic replicators of the gammaherpesviruses Epstein-Barr virus (EBV) and herpesvirus papio (54). The corresponding EBV *ori*Lyt element, known as the downstream component, is essential for replicator function (25, 56). Moreover, deletions in HCMV *ori*Lyt spanning the Y-block abrogated replicator function (68). Therefore, we questioned whether the HCMV Y-block is essential to replicator activity. For these experiments, we made several mutations in and around the Y-block and measured the ability of these *ori*Lyt mutants to mediate replication of plasmids carrying them in a transient transfection-plus-infection assay; results

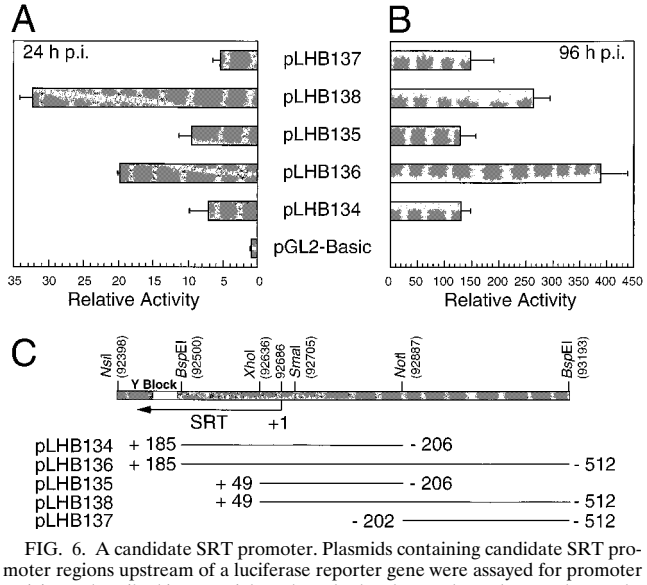

FIG. 6. A candidate SRT promoter. Plasmids containing candidate SRT promoter regions upstream of a luciferase reporter gene were assayed for promoter activity as described in Materials and Methods. The graphs at the top plot results from one representative experiment. The data are expressed as luciferase activity relative to that of the promoterless parent vector, pGL2-Basic, and error bars represent the standard deviations for triplicate wells. (A and B) Plots of luciferase activities obtained from cells harvested at 24 and 96 h p.i., respectively. (C) Physical map of SRT and 5′-flanking regions. The candidate SRT promoter sequences contained in each reporter plasmid are indicated relative to the SRT 5′ end at nt 92686, which was assigned as +1. The Y-block (open box) is indicated.

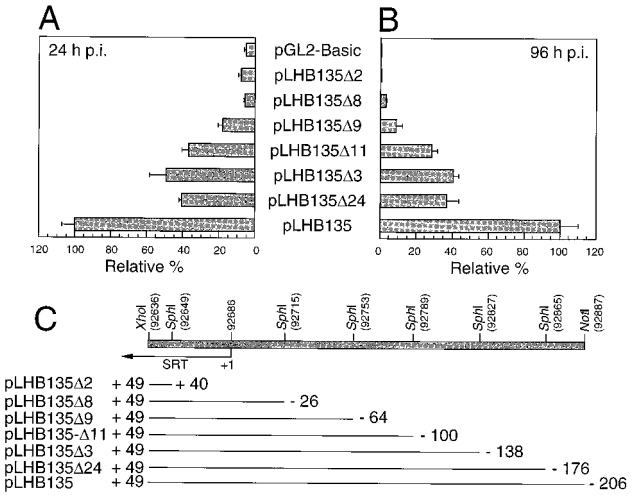

FIG. 7. Deletion analysis of SRT candidate promoter. The indicated reporter plasmids were assayed for promoter activity as described in Materials and Methods. At 24 and 96 h p.i., cells were harvested and assayed for luciferase activity. As in Fig. 6, the graphs at the top plot results from one representative experiment. The data are expressed as percentages of the luciferase activity relative to that of the parent plasmid pLHB135 (set to 100%), and error bars represent the standard deviations for triplicate wells. (A and B) Relative luciferase activities at 24 and 96 h p.i., respectively. (C) The candidate SRT promoter regions contained in pLHB135 and derivatives enumerated as in Fig. 6.

of a representative experiment are shown in Fig. 8. The parental plasmid SP54 served as a positive control (Fig. 8A, lane 1), and the vector pGEM7Zf(−) served as a negative control (Fig. 8A, lane 2). A mutation in which the leftmost 22 nt of the Y-block was deleted and replaced with a *Pst*I site (a net deletion of 16 nt; pYZ9) abolished *ori*Lyt function (Fig. 8A, lane 3). Likewise, substitutions in which the segment deleted in pYZ9 was replaced with random sequence to preserve the spacing of adjacent sequences (pYZ9R2) or replaced so as to produce a net 4-bp insertion (pYZ9R) also were incapable of mediating replication (Fig. 8A, lanes 4 and 5, respectively). For each of the Y-block mutants, full activity was rescued by replacing the mutated region with the wild-type sequence (68). Therefore, we concluded from these results that the Y-block sequence lying at the SRT 3′ end is essential to replicator function.

DISCUSSION

Structure and expression of SRT. Results presented here (i) establish the temporal expression and the structure of SRT, a novel, small *ori*Lyt RNA; and (ii) demonstrate that a conserved DNA sequence motif termed the Y-block, overlapping the variable 3′ end of SRT, is essential to HCMV *ori*Lyt replicator function. SRT was defined as an early transcript by its temporal expression and by its normal expression when viral DNA replication was inhibited by treatment with PFA but not when viral immediate-early protein synthesis was blocked with cycloheximide. Its temporal accumulation was clearly distinct from that of the other *ori*Lyt transcripts. Low-level accumulation of SRT was seen by 2 h after infection. In contrast, the overlapping 6.5-kb RNA was not seen before 24 h p.i. and accumulated only at late times, although it was minimally detectable in RNA from infected cells treated with PFA for 96 h (Fig. 2). UL59 transcripts were detected only under late conditions (33). RT-PCR-mediated cDNA cloning of SRT identified a single 5′ end at around nt 92686 and heterogeneous 3′ ends within a 45-nt window between nt 92431 and 92474, overlapping the conserved Y-block element. The single 5′ end contrasts with UL57 and UL59 transcripts, which have multiple start sites (32, 33). Both the size and the location established by cDNA cloning were consistent with results of Northern analyses, and together these results defined an RNA of about 210 to 255 nt.

SRT was enriched in Northern blots of oligo(dT)-selected RNA preparations, and the oligo(dT)-selected RNA preparations were successfully used for RACE experiments. However, none of the 11 SRT RLM-3′-RACE cDNA clones exhibited polyadenylation, whereas the 3′ ends of all of the equivalently cloned UL59 transcripts were polyadenylated. This finding, together with the lack of a genomic polyadenylation signal in this region and the failure of oligo(dT)-mediated 3′-RACE to amplify SRT 3′ ends, makes a strong argument that SRT is not polyadenylated, unlike most other well-characterized HCMV transcripts. The peculiar 3′-terminal sequence of SRT that includes a stretch (~31 nt) of poly(G) may be the molecular basis for its retention by oligo(dT), because within RNA secondary structures G can stably pair with U (39, 62), and dG · dT pairs can be formed in DNA (26, 59). The failure of conventional oligo(dT)-primed 3′-RACE to work for SRT might be explained by the different temperatures employed; preparation of oligo(dT)-selected RNA was done at room temperature, whereas cDNA synthesis in the conventional 3′-RACE was carried out at 42°C.

The structure and expression of SRT raise several important questions about its genesis. First, it remains to be established whether SRT is expressed via a discrete transcription initiation event or whether the 5′ end is produced by processing or cleavage from a precursor RNA. We have no evidence to support the latter model. The crossing 6.5-kb transcript is expressed with a time course different from that of SRT and does not appear to be spliced in the SRT region. Therefore, it is unlikely that SRT represents a stable intron or other RNA processing intermediate derived from the 6.5-kb RNA. Neither the SRT sequence nor intervening sequences were present at the 5′ ends of UL57 or UL59 transcripts (32, 33), and no other candidate precursor transcripts were seen in Northern blots or PCR experiments. On the other hand, the finding that a fragment encompassing the SRT 5′ end functioned as a promoter in transient-transfection assays of reporter constructs (nt +49 to −206; Fig. 6) is consistent with a discrete SRT transcription unit. Preliminary deletion analysis suggests that the segment between nt +49 and −100 contains a basal promoter and that distal sequences, extending beyond nt −206, augment promoter activity (Fig. 7). The candidate promoter region spans previously noted clusters of reiterated 12-bp (GTGCGCATGCGC) and 15-bp [GGTA$(N)_5$CCACT] sequence elements that are unique to the HCMV *ori*Lyt region (2, 3, 28, 47), as well as a dyad symmetry with some features of an IE2 86-kDa protein binding site [CGG$(N)_8$CCG] (4, 57). However, there is nothing that resembles an upstream TATA box near the SRT 5′ end. The distal upstream region that augmented promoter activity (nt −207 to −512) overlaps a cluster of Sp1 consensus sequences. Deletion analysis suggests that this distal region is critical to *ori*Lyt replicator activity (68). It remains to be determined whether any of these elements contribute to promoter function in the transient-transfection assays. A central question to be addressed in future studies is whether this candidate promoter region directs SRT expression in the context of the viral genome.

Second, the nature of SRT 3′-end formation is also unclear. The heterogeneous 3′ ends could be produced by directed transcription termination or by cleavage of a longer precursor.

FIG. 8. The Y-block is essential for *ori*Lyt function. (A) The abilities of the indicated plasmids to mediate DNA synthesis were assessed in a transient-replication assay as described in Materials and Methods. Equal aliquots of each transfected-cell DNA preparation were digested to completion with *Dpn*I and *Eco*RI, subjected to electrophoresis through a 0.8% agarose gel, and transferred to a Zeta-Probe membrane. The transfer was probed with ^{32}P-labeled pGEM7Zf(−) (pGEM), and the hybridizing fragments were visualized by autoradiography. Only the portion of the blot containing the replicating signals is shown. The positions of the replicated signal (rep'd) and pGEM are indicated. Because of the background replication inherent in this assay, pGEM also was included in each transfection as an internal negative control; note that the amount of pGEM in the pGEM-only transfection (lane 2) was three times that in the other samples (lanes 1 and 3 to 5). Only signals reproducibly higher than the normalized internal pGEM control were scored as positive. (B) Sequences of the wild-type Y-block and the Y-block mutations in pSP54, pYZ9, pYZ9R2, and pYZR. Sequence changes are underlined.

Neither Northern analysis nor RT-PCR experiments detected a candidate precursor (save the 6.5-kb transcript), but we cannot yet rule out this possibility because, if cleavage was rapid and efficient, it might be difficult to detect.

Finally, if SRT synthesis is initiated at the established 5′ end, as the data suggest, which RNA polymerase is responsible for its transcription? Its small size and lack of polyadenylation suggest that polymerase III (PolIII) could be responsible; such small PolIII RNAs have been reported in several virus systems, including adenovirus (20), EBV (5, 37), and HCMV (46). However, preliminary experiments done using HCMV-infected cells suggest that SRT synthesis is sensitive to low concentrations of α-amanitin, consistent with PolII synthesis. In addition, we have found that in the transient-transfection assay system the candidate SRT promoter is responsive to the major immediate-early proteins and other transactivators of HCMV early gene expression (32, 36). Therefore, we think it likely that PolII is responsible for SRT transcription. PolII synthesis of small nonpolyadenylated RNAs is not without precedent, most notably of histone mRNAs and some small nuclear RNAs (9, 21, 43, 49). It remains important to address this question directly.

Does SRT participate in initiation of DNA synthesis? The structure of SRT suggests that it is not a messenger, although we have not examined polysome-associated RNA. The UL60 ORF begins upstream of SRT, spans its entire length, and includes a potential start codon at position 92575, but the UL60 stop codon lies outside of SRT well beyond the established 3′ ends. We found no evidence of splicing within SRT, and therefore it is unlikely that a stop codon from another reading frame is employed. No other ORFs are present in SRT. Another possibility is that an undetected, longer and potentially coding precursor is made. However, the Genetics Computer Group program TESTCODE did not find statistical evidence for protein coding in the SRT region, although the known HCMV coding ORFs that we examined were significantly positive by this test (23).

If SRT is not a messenger, then what is its function? The coincidence of SRT 3′ ends with an essential replicator element suggests the interesting hypothesis that SRT plays a role in initiating, or regulating initiation of, DNA synthesis. The relative complexity of HCMV *ori*Lyt, the apparent lack of an HCMV homolog of HSV-1 UL9, and the requirements for additional, nonhomologous proteins to complement DNA replication all suggest that HCMV may utilize a molecular mechanism to initiate lytic-phase DNA replication that is fundamentally distinct from that employed by HSV-1. Regulation of replicator function by transcriptional control elements and/or transcripts occurs in many systems; several distinct but nonexclusive mechanisms have been established (16). The properties of SRT are consistent with models suggested by this hypothesis: it is transcribed early after infection and in the absence of viral DNA replication, it is not polyadenylated, and it does not appear to be a coding species. It lies in the center of the *ori*Lyt core region defined by insertion mutagenesis, and segments that are essential to *ori*Lyt replicator activity flank it. Most strikingly, the overlapped oligopyrimidine block is the only single-sequence element in HCMV *ori*Lyt yet found to be essential for *ori*Lyt-mediated DNA synthesis. Similar elements are present in the lytic replicators of other CMVs and EBV, which like HCMV lacks an apparent homolog of UL9 (19), but not in the lytic replicator of the betaherpesvirus human herpesvirus 6, which encodes a functional UL9 homolog (34, 35); like the HCMV Y-block, the corresponding EBV sequence is essential to replicator function (56). The role of these sequences in initiation of DNA synthesis is not understood (25).

In one possible model, SRT might cooperate with the Y-block to produce initial strand separation, much as observed in the mitochondrial heavy-strand replicator (15, 67), in which a similar highly conserved element called CSBII promotes the formation of a stable hybrid between crossing, nascent mitochondrial RNA polymerase transcripts and the template strand, displacing the opposite DNA strand and forming a locally opened region coincident with the origin of DNA synthesis. In that system, subsequent cleavage of the transcript by a specific nuclease activity provides the initiating primer for DNA synthesis. This or other initiator transcript mechanisms could explain, at least in part, both the relative complexity of HCMV *ori*Lyt and the lack of a requirement for an initiator protein counterpart to HSV-1 UL9. However, such models predict a requirement for a viral transcription activator(s) in initiation and are consistent with overlapping, essential, specific protein-DNA interactions.

Another obvious prediction of such models is that mutation

of the SRT promoter should correspondingly inhibit replicator function, and, indeed, deletion of the entire 5′ region contributing to SRT expression in transient-transfection assays, extending past nt −512 relative to the SRT 5′ end, abrogated replicator function (68). However, this putative SRT regulatory region overlaps a distal upstream segment that is essential to replicator function, probably independent of SRT expression (68). Deletion of the 5′ end of SRT, including the cap site and upstream ~200 nt, does not destroy replicator activity (68), but the data presented here indicate that such deletions uncover cryptic start sites in transient-transfection assays (Fig. 6, pLHB137). Moreover, because *ori*Lyt-mediated replication produces tandem oligomers via a rolling circle (3), an initiator transcript mechanism might not display a quantitative relationship between transcription and replicator activities. Therefore, because all replicator assays have been done transiently, this result is not inconsistent with initiator transcript models. A further obstacle to interpretation of these results is that promoter mutations might simultaneously affect transcription and replication by independent pathways. Thus, although such models should be testable, unequivocal tests may require approaches directed towards elucidating biochemical details of initiation.

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