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Boundaries and Structure of Human Cytomegalovirus *ori*Lyt, a Complex Origin for Lytic-Phase DNA Replication

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We have localized a *cis*-acting sequence that promotes initiation of lytic-phase DNA replication (*ori*Lyt) within the *Hin*dIII D fragment of the human cytomegalovirus (HCMV) AD169 genome and investigated its sequence requirements by testing the ability of plasmid constructs to mediate DNA replication in a transient transfection-plus-infection assay. Replication of plasmids containing HCMV *ori*Lyt required at least the virus-specified DNA polymerase activity supplied by HCMV infection of transfected cells and was autonomous in that it did not result from recombination with the virus genome. Progeny molecules in the transient assay were high-molecular-weight tandem oligomers, which is consistent with predictions of a rolling-circle model. Experiments testing subclones of *Hin*dIII-D defined a core 2.4-kbp region containing elements required for *ori*Lyt function that extended rightward from around 1.0 kbp upstream of UL57 near the middle of the long unique component of the virus genome. Sequences flanking this core also were needed for full activity. The defined region contains at least four clustered sets of repeated sequence elements identical to or candidate counterparts of elements present in the corresponding cytomegalovirus Colburn lytic-phase replication origin. These elements are novel in that they apparently do not correspond to previously characterized motifs. Also present are multiple copies of elements similar to known binding sites for the transcription factors ATF/CREB, MLTF/USF, and Sp1. Preliminary deletion analysis suggests that multiple components within the boundaries of *ori*Lyt cooperate to enable initiation of HCMV lytic-phase DNA synthesis.

Our laboratory recently identified by transient transfection assay a minimal 1.3-kbp sequence within the genome of simian cytomegalovirus (SCMV) Colburn that directs the replication of plasmid molecules when the virus-coded DNA polymerase and other necessary *trans*-acting factors are supplied by infection; we also obtained evidence that a corresponding region of the human cytomegalovirus (HCMV) genome directs lytic-phase DNA replication (4). In both SCMV and HCMV, this element, here designated *ori*Lyt, is situated immediately 5′ of the gene encoding the single-stranded DNA-binding protein homologous to herpes simplex virus type 1 (HSV-1) UL29 (1, 2, 32). Independently, Hamzeh et al. (25), using a novel approach, obtained evidence for initiation within this segment of both HCMV and SCMV genomes. The results of these two studies were consistent, providing evidence that *ori*Lyt represents an authentic origin of genomic DNA replication. The identification of this key component of the replication machinery and the demonstration that it can mediate DNA replication in a transient assay have made possible its systematic molecular dissection. Further, in the absence of more-traditional genetic data, the identification of *ori*Lyt should make it possible to find the complete set of virus genes required for viral DNA replication by using the cotransfection method developed by Challberg and colleagues (11, 39, 55).

Here, we describe experiments that further support the hypothesis that this segment is an authentic origin for HCMV lytic-phase DNA replication and elucidate the general structure of this origin as a first step toward defining required functional elements. Results demonstrate that HCMV *ori*Lyt spans more than 2.4 kbp of sequence containing numerous reiterated and inverted sequence elements, including both known consensus transcription factor recognition sites and previously unrecognized motifs, as well as other interesting structural features. Preliminary deletion analysis suggests that several of these sets of repeated elements are required for or contribute to *ori*Lyt function and argues that little of the 2.4-kbp sequence is dispensable.

MATERIALS AND METHODS

Virus and cells. Human foreskin fibroblasts (HF cells) were used for all experiments. Their preparation, passage, and infection were as previously described (22, 23). The apparent transfection efficiencies of several independently prepared HF lines were compared, and the best were selected for use in these studies. Cells used for transfection experiments were low to moderate passage number. HCMV Towne and AD169, provided by W. Gibson, were stored as frozen stocks, passaged at low multiplicity, and periodically checked by restriction analysis.

Recombinant plasmids. The 23.8-kbp HCMV AD169 *Hin*dIII D fragment was purified by agarose gel electrophoresis and ligated into the *Hin*dIII site of pGEM-7Zf(−) (Promega, Madison, Wisc.) to make pSP24. The *Pst*I, *Pvu*II, *Bam*HI, and *Bgl*II subclones of *Hin*dIII-D were constructed by treating pSP24 with *Pst*I, *Pvu*II, *Bam*HI, or *Bgl*II; purifying the fragment delimited by the indicated restriction sites (see Fig. 1B and Results) within *Hin*dIII-D; and ligating the purified fragment into the corresponding site of pUC18 (*Pst*I, *Pvu*II) or pGEM-7Zf(−) (*Bam*HI, *Bgl*II) to generate pSP31, pSP33, pSP34, and pSP37, respectively. The *Pvu*II-*Bgl*II subclone pSP38 was constructed by treating pSP24 with *Pvu*II and *Bgl*II, purifying the 5.9-kbp fragment extending

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from nucleotide (nt) 89,795 to 95,671, and ligating the purified fragment into pGEM-7Zf(−) treated with *Sma*I and *Bam*HI.

Several independent *Pvu*II-*Kpn*I subclones were constructed by treating pSP38 with *Kpn*I and purifying the 5.1-kbp fragment delineated by the *Kpn*I restriction site at nt 94,860 and the adjacent vector *Kpn*I site. The purified fragment then was ligated in both orientations either into *Kpn*I-treated pGEM-7Zf(−), producing pSP50 and pSP51, or into the *Kpn*I site of a pGEM-7Zf(−)-derived vector modified to remove its *Sph*I site, producing pSP55 and pSP56.

A deletion between *Bss*HII sites at nt 91,005 and 91,736, labeled pSP39, was made by treating pSP38 with *Bss*HII and religating. The internal *Bam*HI deletion pSP53 was constructed by treating pSP38 with *Bam*HI, removing the segment between the indicated restriction sites (see Fig. 8), and religating. The deletion between *Bst*EII sites (pSP57) was made by treating pSP51 with *Bst*EII, removing the segment extending from nt 93,361 to 93,513, and religating. The internal *Sph*I deletion subclone pSP62 was constructed by treating pSP56 with *Sph*I, excising the segment between the indicated restriction sites, and religating.

The *Nhe*I-*Kpn*I and *Aat*II-*Kpn*I subclones pSP54 and pSP60 were constructed by treating pSP51 with *Xba*I plus *Nhe*I or with *Aat*II, respectively, removing the segment between the restriction site(s) within the inserted fragment and the adjacent corresponding site in pGEM-7Zf(−) multiple cloning site, and religating. The *Pvu*II-*Sac*I subclone pSP49 was made by treating pSP38 with *Sac*I, removing the segment between the *Sac*I site at nt 93,715 and the *Sac*I site in pGEM-7Zf(−), and religating. The *Nhe*I-*Bgl*II subclone pSP52 was constructed by treating pSP38 with *Xba*I and *Nhe*I, excising the segment between the *Nhe*I site at nt 90,504 and the pGEM-7Zf(−) *Xba*I site, and religating. The *Aat*II-*Sac*I clone pSP65 was made by excising the segment between the *Sac*I site at nt 93,715 and the *Sac*I site in the pGEM-7Zf(−) multiple cloning site from pSP60, and religating.

Bidirectional exonuclease III deletions were done essentially as described previously (4) by using the Erase-a-base system (Promega) after the following plasmids were cut with the indicated enzymes: pSP54 with *Eco*RI (pSP68), pSP63 with *Bss*HII (pSP69), pSP38 with *Bam*HI (pSP70), and pSP51 with *Bst*EII (pSP77). The extent of each resulting deletion was determined by sequencing selected clones.

Transient transfection assays. Constructs were assayed for their abilities to direct replication following DEAE-dextran transfection essentially as described previously (4, 47), with a few modifications. Briefly, freshly confluent HF cells were split 1:3 onto 6-cm dishes 24 h prior to transfection. Transfection mixtures contained about 1 pmol of test plasmid, and transfection was carried out for 4 h and followed by a 1-min treatment with phosphate-buffered saline containing 10% (vol/vol) dimethyl sulfoxide. In some experiments, equimolar pGEM-7Zf(−) was included as an internal negative control. Transfected cells were infected with 10 to 100 PFU per cell 24 h after transfection. DNA was prepared 96 h after infection by adding 350 μl of cell lysis buffer (5.0 mM Tris-HCl [pH 8.0], 100 mM EDTA, 400 mM NaCl, 0.5% sodium dodecyl sulfate, 2% Sarkosyl) containing 200 μg of proteinase K per ml directly to rinsed culture dishes, swirling to release the lysate, and incubating for 2 to 4 h at 60°C. The DNA was then purified by successive phenol and chloroform extractions and precipitated. Redissolved preparations were quantitated by fluorometry. After the indicated restriction enzyme treatment and agarose gel electrophoresis, transfer to a nylon filter (ZetaProbe; BioRad, Richmond, Calif.) was done by using the alkaline method per the supplier's protocol. Because complete digestion with *Dpn*I is crucial to interpreting results, restriction digestions were checked in parallel digestions or by adding nonreplicating plasmid DNA to transfection mixtures as internal controls. Transfers were probed with radiolabeled pGEM-7Zf(−). All transfection assays were repeated at least twice.

Nucleotide sequencing and analysis. When necessary to establish or confirm the structure of deletion constructs, nucleotide sequencing was done by the dideoxy method, using Sequenase (U.S. Biochemicals, Cleveland, Ohio) and synthetic oligonucleotide primers obtained from the Wadsworth Center for Laboratories and Research Molecular Biology Core Facility. Sequence data were compared with the published HCMV nucleotide sequence (accession number X17403 [14]) by using the University of Wisconsin Genetics Computer Group GCG sequence analysis package (16).

RESULTS

Localization of the HCMV counterpart to SCMV *ori*Lyt. Previous results suggested that a functioning HCMV replication origin is situated within strain Towne *Hin*dIII-A near the center of U_L (4; Fig. 1A, lane 7). Inspection of the HCMV nucleotide sequence from this region revealed notable similarities to that of SCMV *ori*Lyt (detailed in Discussion) in a position corresponding to the position of SCMV *ori*Lyt immediately upstream of the UL57 open reading frame. However, several independent subclones containing the HCMV Towne *Bam*HI K fragment, extending about 2 kbp 5′ of UL57 and including the identified sequence similarities, failed to direct replication in our assays (4). Therefore, to better locate the HCMV origin, we first confirmed these results by using the corresponding *Hin*dIII D and *Bam*HI M fragments from the sequenced prototype HCMV strain AD169 and subsequently tested a series of subclones from *Hin*dIII-D by using the standard transient assay described in Materials and Methods. As anticipated, the strain AD169 *Hin*dIII D fragment mediated replication (Fig. 1A, lane 6), whereas AD169 *Bam*HI-M did not (Fig. 1A, lane 2). However, subclones extending upstream of UL57 beyond the *Bam*HI sites at nt 93,361 and 93,513 retained the ability to direct replication. The *Pvu*II fragment spanning nt 89,795 to 102,278, the *Pst*I fragment from nt 85,373 to 98,962, and the *Bgl*II fragment from nt 87,269 to 95,671 all replicated without noticeably lower efficiency than *Hin*dIII-D (Fig. 1A, lanes 3 to 5). Not shown in Fig. 1A is the pGEM-7Zf(−) negative control, which was routinely included. The minimal fragment defined by these initial experiments, extending 5.9 kbp from the *Pvu*II site at nt 89,795 to the *Bgl*II site at nt 95,671, also replicated as efficiently as *Hin*dIII-D (shown in Fig. 3 and 4).

Lytic-phase HCMV genomic DNA replication requires an as-yet-undetermined set of virus-coded genes, including a DNA polymerase (26, 36). To determine whether the observed plasmid-mediated DNA replication depends on virus gene expression, we tested the abilities of plasmids containing this region of the HCMV genome to direct replication without subsequent virus infection. HF cells transfected with a plasmid containing the replication-competent *Bgl*II fragment (pSP37; Fig. 2, lane 3), but not infected did not detectably replicate the plasmid (Fig. 2, lane 5). Thus, replication required a viral contribution. Moreover, inhibiting virus genomic DNA synthesis by treating pSP37-trans-

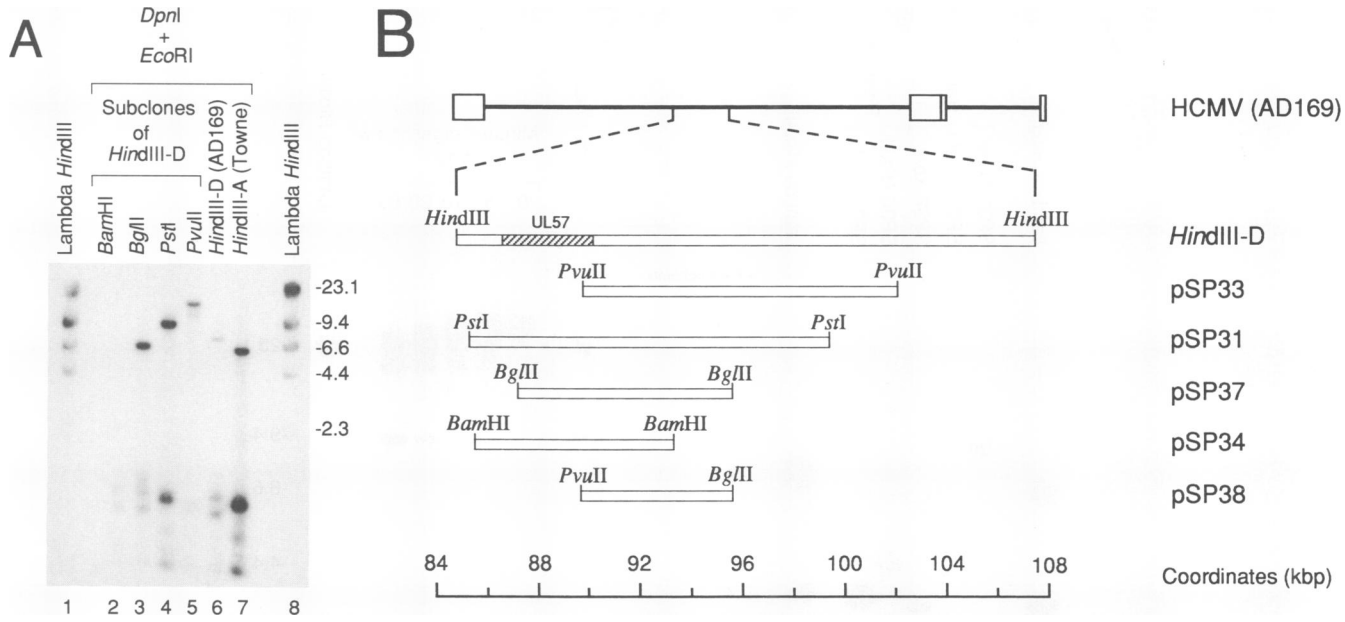

FIG. 1. Location of HCMV *ori*Lyt. (A) Transient transfection assays of HCMV AD169 *Hin*dIII-D and subclones. The abilities of the indicated plasmid constructs to mediate replication were assessed in a transient transfection assay as described in Materials and Methods. Transfected-cell DNA was treated with the restriction enzymes *Dpn*I and *Eco*RI, and 1 μg of each sample was subjected to electrophoresis through a 0.8% agarose gel and transferred to a Zeta-probe filter. The transfer was probed with ^{32}P-labeled pGEM-7Zf(−), and the hybridizing fragments were visualized by autoradiography. The plasmid used to transfect cells for each sample is indicated above each lane. Lanes 1 and 8 contain bacteriophage lambda DNA digested with *Hin*dIII as a size marker; the sizes of marker fragments (in kilodaltons) are given on the right. (B) Physical locations of HCMV AD169 *Hin*dIII-D and of the cloned *Hin*dIII D subfragments assayed for panel A. Clone designations are given to the right for each fragment. The position of UL57 within *Hin*dIII-D is indicated by cross-hatching. The boxes drawn within the HCMV genome indicate the positions of terminal repeated sequences. Nucleotide coordinates for cloned fragments are listed in the text.

fected and infected cells with phosphonoformic acid, a selective inhibitor of the virus-specified DNA polymerase (27), also eliminated detectable plasmid DNA replication (Fig. 2, lane 4). This finding demonstrated that replication is not activated solely by a virion factor and argues that the replication of these transfected plasmids is mediated directly or indirectly by the viral DNA polymerase. These results are consistent with those of previous studies of SCMV *ori*Lyt (4).

Products of *ori*Lyt-mediated replication. One possible explanation for polymerase-dependent replication of plasmids containing this element was that it promoted high-frequency recombination with virus genomic DNA; in that case, DNA replication could be driven by a separate viral genomic origin. Therefore, the structures of plasmid-derived replication products were examined to determine whether replication was autonomous (Fig. 3A). In the experiment whose results are shown in Fig. 3, DNA prepared from HF cells transfected with a replication-competent plasmid containing the *Pvu*II-to-*Bgl*II fragment (pSP38) and then infected was treated with *Dpn*I, to distinguish replicated from unreplicated molecules, plus (i) no additional enzyme (Fig. 3A, lane 2); (ii) *Hin*dIII, which does not cleave pSP38 but does cut flanking sites within the virus genome (Fig. 3A, lane 3); or (iii) *Xba*I, which cleaves pSP38 at a unique site within the vector but not within the insert (Fig. 3A, lane 4). Digested DNA was then probed with ^{32}P-labeled vector to distinguish input plasmid DNA from virus genomic fragments following Southern transfer. The bulk of uncut or *Hin*dIII-treated replication products migrated as unresolved high-molecular-weight species at the linear discontinuity, readily distinguishable from input supercoiled, open-circle, or linear

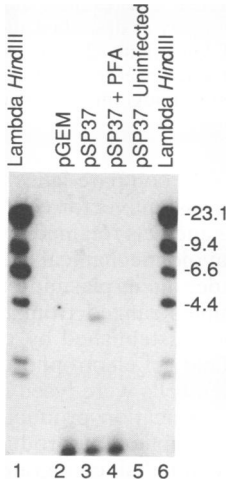

FIG. 2. *ori*Lyt-mediated replication requires the activity of a virus-coded factor(s). HF cells were transfected with pGEM-7Zf(−) (lane 2) or with pSP37 containing the 8.4-kbp *Bgl*II fragment (lanes 3 to 5), and all except the sample corresponding to lane 5 were infected 24 h later as described in Materials and Methods. Phosphonoformic acid was added to the sample for lane 4 to a final concentration of 100 μg/ml at the time of infection. DNA subsequently prepared from each sample was digested with *Dpn*I and *Eco*RI, and 1 μg of each digested DNA preparation was analyzed as described in Materials and Methods. Shown is a photograph of the resulting autoradiogram. Positions of bacteriophage lambda *Hin*dIII fragments run in parallel are indicated. Numbers on the right are molecular sizes in kilodaltons.

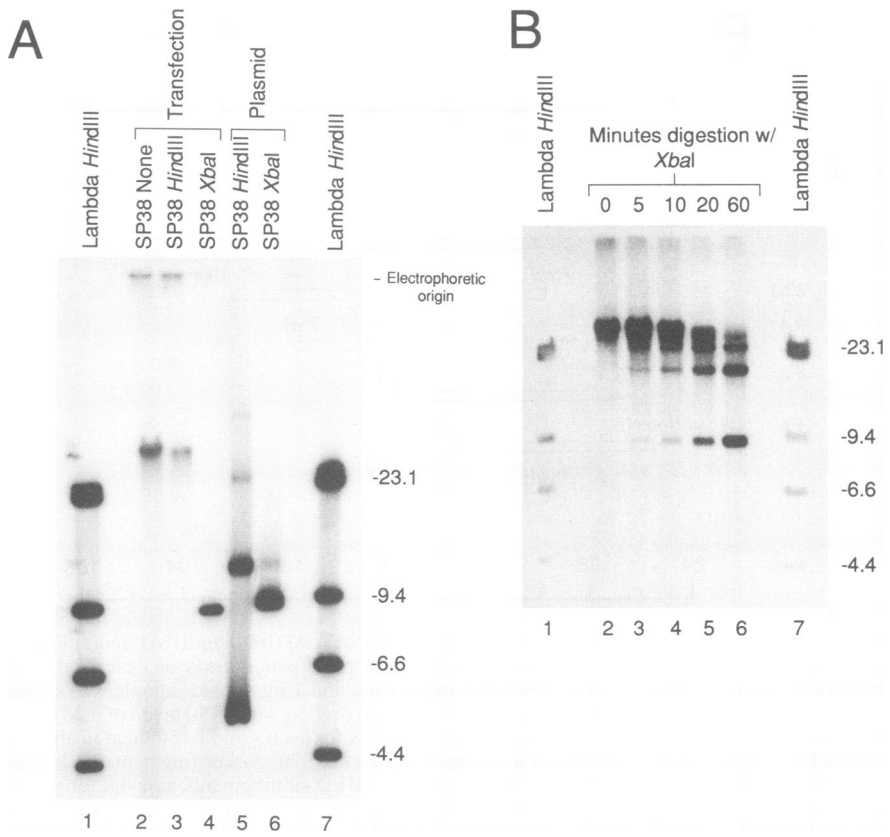

FIG. 3. Structure of pSP38 replication products. (A) Digestion to completion with *Hin*dIII or *Xba*I. HF cells were transfected with pSP38 and infected, and DNA was prepared as described in Materials and Methods. Equal aliquots of transfected-cell DNA were digested to completion with *Dpn*I and the indicated enzymes (lanes 2 to 4; see text), subjected to electrophoresis through a 0.6% agarose gel, transferred to a nylon filter, and probed with ^{32}P-labeled vector DNA. Untransfected SP38 plasmid DNA was treated with either *Hin*dIII (lane 5) or *Xba*I (lane 6) and run in parallel. The resulting autoradiogram is shown. (B) Partial digestions. DNA was purified from HF cells transfected with pSP38 for the transient assay as described above. A 5-μg sample of transfected-cell DNA was first digested to completion with *Dpn*I and then incubated with 5 U of *Xba*I (lanes 2 to 6). Aliquots (1 μg each) were removed at the indicated intervals, and the digestion was stopped by adding gel loading buffer and heating. Samples were examined by electrophoresis through a 0.6% agarose gel and Southern analysis as in panel A, and the resulting autoradiogram is shown. Flanking marker lanes (in kilodaltons) for both panels are *Hin*dIII-treated lambda DNA.

plasmid DNA (Fig. 3A, compare lanes 2 and 3 with lanes 5 and 6). Some *Dpn*I-resistant vector-containing DNA was too large to enter the gel and was retained at the origin. Although no special effort to avoid mechanical shearing during sample preparation was made, uncut plasmid-containing DNA fragments in these samples ranged from approximately 50 to more than 200 kbp as established by contour-clamped homogenous-electric-field gel electrophoresis versus a lambda genomic ladder; when cells were lysed in an agarose plug to eliminate shearing, replication products were much larger (3). *Xba*I digestion of replication products (Fig. 3A, lane 4) produced a single band that comigrated with the 8.9-kbp *Xba*I-linearized input plasmid (Fig. 3A, lane 6) and greatly reduced that fraction of the replication products that failed to enter the gel when uncut or when treated with *Hin*dIII. Similar results were obtained with other replication-competent plasmids and other restriction enzymes (not shown). In no case did we find evidence that recombination products contributed detectably to plasmid replication. On the basis of these findings and the results of Hamzeh et al. (25), we inferred that the mechanism of replication is likely to be similar or identical to that of the viral lytic-phase, and therefore, we designated this region *ori*Lyt.

Herpesvirus genomic DNA replication is thought to generate a tandem array of daughter genomes, perhaps via a rolling-circle mechanism (reviewed in reference 12). To determine whether the structures of HCMV *ori*Lyt-containing plasmid replication products are consistent with such a mechanism, transient-assay DNA preparations were cleaved partially with restriction enzymes that recognized a unique vector site and examined after Southern transfer (Fig. 3B). This treatment produced a ladder of fragments with mobilities estimated to be integer multiples of unit-length linear molecules, i.e., 1N, 2N, 3N, etc. In the example shown, HF cells were transfected with the 8.9-kbp plasmid SP38 (Fig. 3B, lanes 2 to 6), and transfected-cell DNA preparations were cut with *Xba*I. At least six species were resolved on 0.6% agarose gels. Increasing digestion progressively shifted the products to the unit-length species, and complete digestion reduced the products entirely to unit monomers (Fig. 3A, lane 4). Thus, *ori*Lyt-mediated plasmid replication products appear to be tandem arrays. Unit-length linear or circular *Dpn*I-resistant species were not reproducibly detected at 96 h after infection. However, we have not examined the structures of replication products at earlier times or in the presence of replication inhibitors.

Boundaries of *ori*Lyt. To further define the boundaries of *ori*Lyt, we tested a series of deletion constructs for their

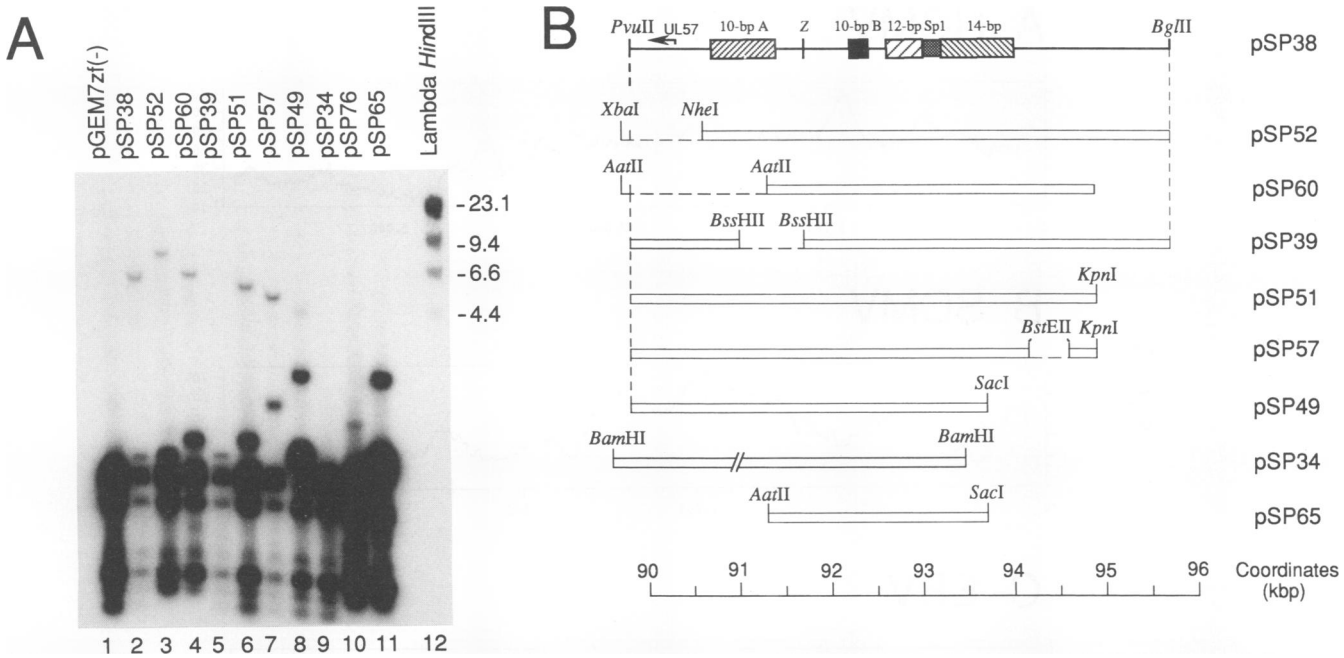

FIG. 4. Boundaries of HCMV *ori*Lyt. (A) Transient transfection assays of representative deletion constructs. Assays were carried out as described in Materials and Methods, and 1 μg of DNA was cut with *Dpn*I plus *Eco*RI. Shown is an autoradiogram following hybridization with the vector probe. Plasmid constructs used to generate the samples, described in the text, are indicated at the top of each lane. Molecular sizes (in kilodaltons) are given at the right. (B) Summary of the physical locations of boundary deletion constructs and their relations to clusters of repeated sequence elements. These elements are described in the text and detailed in Fig. 6.

abilities to mediate replication in the transient assay; we started with the *Pvu*II-to-*Bgl*II fragment (Fig. 4A). On the left, a deletion to the *Nhe*I site at nt 90,253 (pSP52; lane 3) replicated without noticeable defect, and a deletion to the *Aat*II site at nt 91,321 replicated with a severalfold reduction in activity (pSP60; lane 4). However, a deletion between the *Bss*HII sites at nt 91,005 and 91,736 (pSP39; lane 5) abolished replication activity in our standard assay. We therefore inferred the minimal leftward boundary to be situated between the *Aat*II site at nt 91,321 and the *Bss*HII site at nt 91,736. On the right side of *ori*Lyt, a deletion to the *Kpn*I site at nt 94,860 (pSP51; lane 6) failed to reduce activity, as did a 423-bp deletion between *Bst*EII sites at nt 94,168 and 94,591 (pSP57; lane 7). A deletion to the *Sac*I site at nt 93,715 (pSP49; lane 8) produced a replication-positive but very defective *ori*Lyt. Deletion to the *Bam*HI site at nt 93,361 inactivated *ori*Lyt, as expected from previous results (lane 9). Therefore, the minimal rightward boundary probably lies between the *Sac*I site at nt 93,715 and the *Bam*HI site at nt 93,361. We note that deletions around either boundary are progressively defective. Furthermore, plasmid clones containing the 2.4-kbp *Aat*II-to-*Sac*I fragment (pSP65; lane 11) or the similar *Sal*I fragment extending from nt 91,286 to 93,727 (pSP76; lane 10) and combining the individually defined rightward and leftward boundaries did not detectably replicate. Sequences outside these minimal boundaries clearly contributed to *ori*Lyt function as measured by the transient assay. Thus, the boundaries are not sharply defined but rather locate a core region containing elements required for *ori*Lyt function. The physical relationships of deletions defining the flanking boundaries of *ori*Lyt to various structural features described below are diagrammed in Fig. 4B.

Structural features of HCMV *ori*Lyt. To aid functional analysis of *ori*Lyt, we searched the published nucleotide sequence (14) for reiterated and inverted elements and for other features that might contribute to *ori*Lyt activity. The overall base composition of this region (about 62% G+C) is similar to that for the entire genome, but it is notably asymmetric (Fig. 5A). At the leftward boundary is a comparatively A+T-rich segment (up to 70% A+T within some 50-bp windows) located between nt 91,100 and 91,300. An extremely G+C-rich segment extends from around nt 93,000 toward the rightward boundary. Lytic-phase origins of cytomegalovirus Colburn and of Epstein-Barr virus (EBV) also have asymmetric base distributions (Fig. 5B and C, respectively) with an extended G+C-rich segment at one end, suggesting that this asymmetry may represent a conserved physical structure.

The region within and around the above-described HCMV *ori*Lyt boundaries contains numerous repeated motifs, including known transcription factor recognition sequences. Locations of identified repeated and inverted sequences and other discussed features are summarized in Fig. 6; some of these elements in similar forms were described by Hamzeh et al. (25). To our knowledge, five of the identified reiterated sequences do not match previously defined protein-binding sites or structural elements and may be novel (Table 1). Moreover, each of these sets of novel reiterated elements is clustered exclusively within a short segment of *ori*Lyt. For simplicity, domains containing clusters of repeated sequences are designated by the respective element in diagrams relating deletion constructs to these features. In the left half of *ori*Lyt, the 10-bp sequence 5′-ACGGTGTTTT-3′ is present 10 times, allowing one mismatch, within a 750-bp segment of *ori*Lyt. It is situated about equally on both strands, and in several instances, opposite-strand pairs of this 10-bp repeat form a dyad element. This sequence is present elsewhere in the HCMV genome but not in extensive

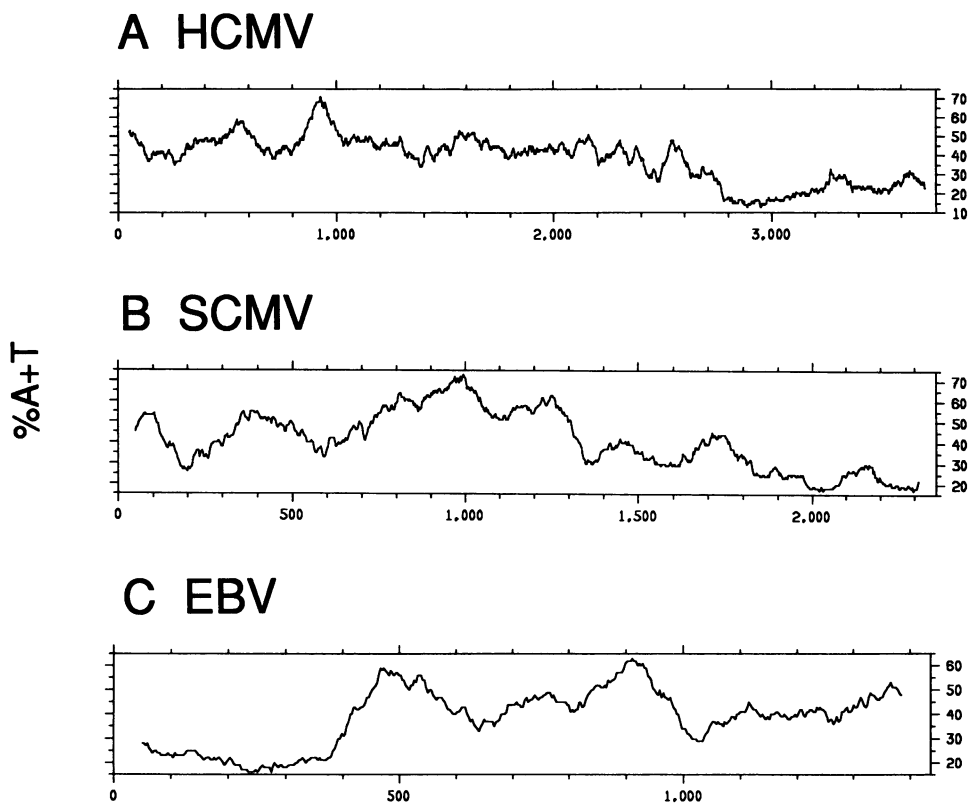

FIG. 5. Asymmetric base distribution within *ori*Lyt. The percent A+T across HCMV *ori*Lyt from nt 90,260 to 94,020 and the published nucleotide sequences of lytic-phase replication origins of cytomegalovirus Colburn (4) and EBV (5, 24) were determined by using the GCG program WINDOW, with the window parameter set to 100 nt and the shift set to 3; the resulting statistics were plotted by using STATPLOT (16).

clusters. A second 10-bp motif, 5′-CGTAGCGAGC-3′, is present in the published sequence near the center of *ori*Lyt as two pairs of overlapping direct repeats, with each pair located between and overlapping inverted pairs of ATF/CREB sites. A few other copies of this 10-bp motif are present elsewhere in the HCMV genome but not as closely spaced direct repeats. Seven regularly spaced direct repeats of the 12-bp consensus 5′-GTGCGCATGCGC-3′, containing *Fsp*I and *Sph*I restriction sites, are situated within a 290-bp segment of *ori*Lyt. Only one imperfect additional copy of this sequence is found elsewhere in the viral genome. Four of the seven 12-bp repeats lie immediately adjacent to a 15-bp repeated sequence, 5′-CGGTAWWWWWCCACT-3′, forming a 27-bp repeated unit. A fifth copy of the 15-bp sequence is found on the opposite strand 3′ of the direct repeats (Table 1). Because both the 12- and 15-bp repeats occur independently, here they are defined separately. No other copies of this 15-bp repeat that allows only one mismatch are present in the HCMV genome. Finally, nine copies of another, more loosely defined 14-bp consensus sequence, 5′-GGGRYCCCTCSCCC-3′, are present within a G+C-rich region at the right end of *ori*Lyt. This reiterated sequence is characterized by pyrimidine tracts of 10 nt or more. Some of these 14-bp repeats are similar to various NFκB consensus definitions.

Most abundant among the sequences within *ori*Lyt that correspond to sites recognized by known transcription factors are 14 copies of the ATF/CREB sequence 5′-ACGTCA-3′ (38, 41) plus additional related sequences; 10 copies of the MLTF/USF core sequence 5′-CGTGAC-3′ (10, 45), several of which include more-extensive matches to MLTF-binding sites; and 11 copies of the Sp1 motif 5′-CCGCCC-3′ (8). With the exception of some clustered ATF/CREB and Sp1 sites, most of these elements are interspersed throughout *ori*Lyt. Also present are candidate TATA, CCAAT, and polyadenylation sequences. Other potential recognition sites, too numerous to catalog here, for various other described transcription factors were found by using the program SIGNALSCAN with the mammalian data base (43) and using the Ghosh database (21) with the UWGCG program FIND (16).

Two distinct, large, but imperfect elements with dyad symmetry are located near the right boundary of *ori*Lyt. The first (dyad A) extends 114 bp from nt 93,097 through nt 93,210, and, when drawn as a hairpin, pairs 45 of 54 nt in the stem (Fig. 7A). The outer and inner portions of the dyad arms are more similar than the central segment. Although present only once in the published nucleotide sequence, this dyad or a closely related sequence is variably repeated in the HCMV AD169 genome, and the additional copies may be spontaneously deleted following cloning (3). A second large palindromic sequence (dyad B) extends 120 bp from nt 93,395 to 93,515 and matches 41 of 56 nt in its dyad arms (Fig. 7B). As with the other large palindromic element, a central segment of the dyad arms shows relatively poor similarity. Numerous smaller dyad symmetries were also found. Perhaps most notable of these was a 20-bp sequence

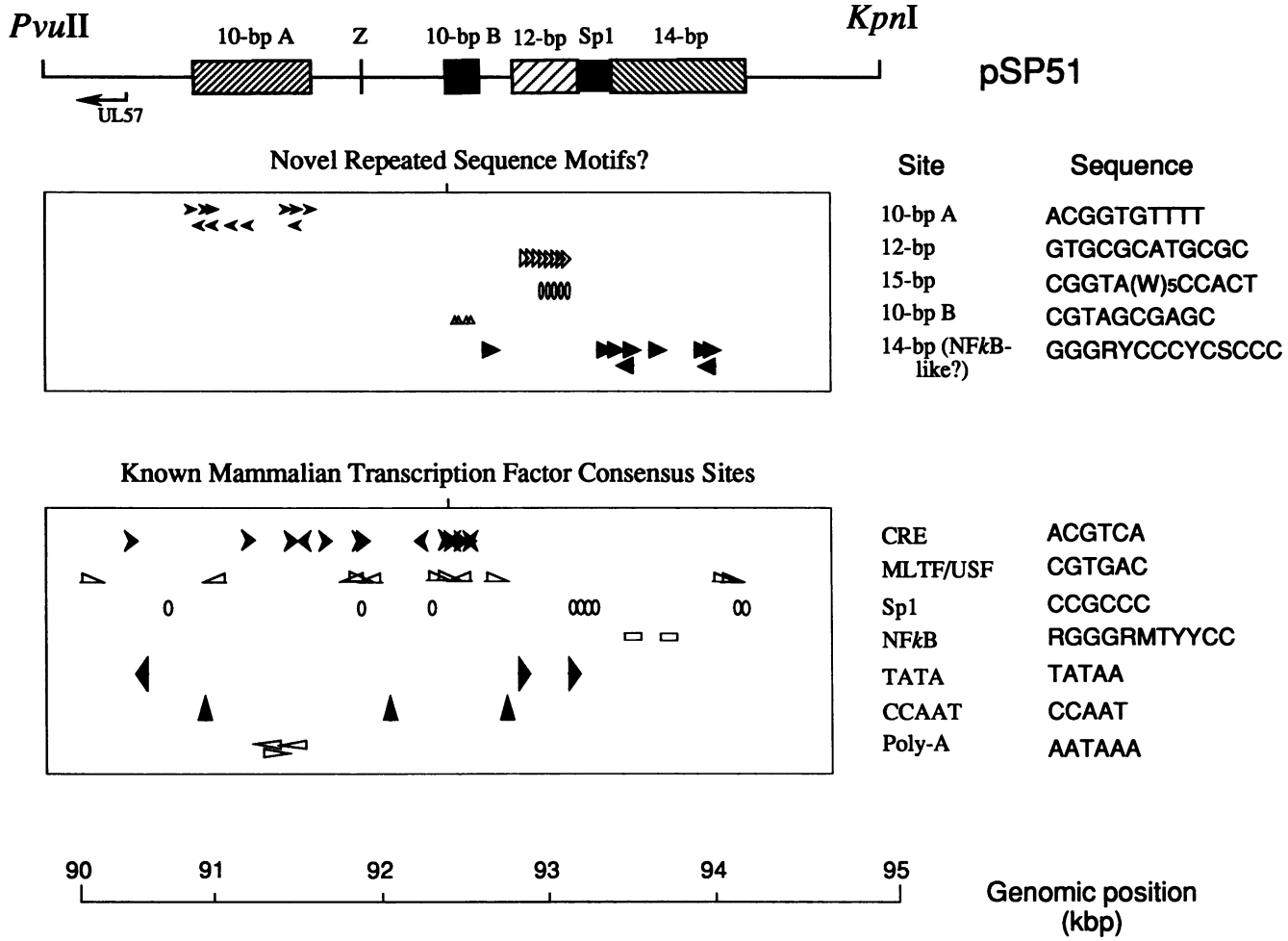

FIG. 6. *ori*Lyt sequence elements. Discussed sequence features are diagrammed to show the locations of clustered novel elements and the juxtapositions of transcription factor consensus sequences. Individual repeated elements are indicated by symbols labeled on the right; the respective consensus sequences are also listed. Shaded boxes within the *Pvu*II-to-*Bgl*II fragment show the positions of domains defined by the clustered novel elements. The scale at the bottom shows approximate genomic coordinates.

extending from nt 91,632 through 91,651 and containing an MLTF/USF site.

Deletions within the *ori*Lyt core. To search for dispensable segments within the central 2.4-kbp *ori*Lyt region, we constructed a series of internal deletions across *ori*Lyt and tested their abilities to direct DNA replication in the transient assay (Fig. 8A). A clone with a 745-bp deletion between the *Bss*HII sites at nt 91,005 and 91,750 (pSP39) inactivated *ori*Lyt, as described above. This deletion removed the relatively A+T-rich segment between nt 91,000 and 91,200 as well as several copies of the 10-bp repeat, several CRE motifs, and a palindrome containing the MLTF/USF consensus (5′-TGTTGGTCACGTGACCATCA-3′) but left six copies of the 10-bp repeat. A 218-nt exonuclease III deletion extending from nt 91,667 to 91,884 in the *Aat*II-to-*Kpn*I fragment inactivated *ori*Lyt (pSP69). This deletion removed a slightly A+T-rich region containing candidate CRE, Sp1, and CAAT elements and a 24-nt stretch of alternating purine and pyrimidine residues. We have not ruled out the possibility that sequences to the left of the AatII site that are missing from pSP69 might complement this defect. A smaller deletion from the *Aat*II-*Kpn*I fragment, between the *Bss*HII sites at nt 91,736 and 91,750 and removing only 14 bp within the short stretch of alternating purine and pyrimidine residues (pSP63), did not inactivate *ori*Lyt.

A 282-nt exonuclease III deletion extending from nt 92,110 to 92,393 (pSP68) destroyed *ori*Lyt function. This deletion removed the clustered CRE core consensus elements and all four associated 10-bp 5′-CGTAGCGAGC-3′ direct repeats as well as a pair of MLTF/USF core sequences. A construct missing the sequence between *Sph*I sites at nt 92,576 and 92,392 retained activity (pSP62), although reduced several-fold. This deletion removed six of seven 12-bp direct repeats and three of five copies of the 15-bp repeats. Similarly, a 152-bp deletion between the *Bam*HI sites at nt 93,361 and 93,513 that removed the 120-bp dyad element (pSP53) reduced but did not eliminate plasmid replication. However, a 725-bp exonuclease III deletion spanning nt 93,197 to 93,922 and removing a portion of the 114-bp dyad along with most of the G+C-rich region, including the 14-bp repeats (pSP70), did not replicate. A construct deleted between *Bst*EII sites at nt 94,168 and 94,591 (pSP57) replicated without apparent defect. Finally, exonuclease III deletions between nt 93,930 and the *Kpn*I site at nt 94,860 (e.g., pSP77) also replicated efficiently. Figure 8B summarizes results of these preliminary deletion experiments.

TABLE 1. Locations of novel repeated sequence elements within HCMV *ori*Lyt[a]

Consensus	Top strand	Bottom strand
ACGGTGTTTT	90,698: AGAAA ACGGcGTTTT CTGTC	90,679: ACGTG AAAACACCGT CCCTA
(allowing 1 mismatch)	90,768: ACCGT ACGGTGTTTg TGACC	90,758: CCCCG AAAACACCGT ACGGT
	90,832: CGAAC ACGGTGTTTT TAAAT	90,971: CCGAC AAAACgCCGT ACAAG
	91,274: GGGGA ACGGTGTTgT GTGTC	91,256: GCCAT gAAACACCGT GATGG
	91,341: GGTGC cCGGTGTTTT TATTC	
	91,433: TCATA ACGGgGTTTT GGGTA	
CGTAGCGAGC	92,230: GGTGA CGTAGCGAGC GTAGC	None
(allowing 1 mismatch)	92,239: GCGAG CGTAGCGAGC TACGT	
	92,297: GATGA CGTAGCGAGC GAAGC	
	92,306: GCGAG CGaAGCGAGC TACGT	
GTGCGCATGCGC	92,568: TTTCC GTGCGCATGCGC AGTCG	None
(allowing 1 mismatch)	92,641: CTCGA GTGCGCATGCGC CGGGG	
	92,707: CCCCG GgGCGCATGCGC GGGCC	
	92,745: TCGCC cTGCGCATGCGC CGGTA	
	92,781: GTGTC GTGCGCATGCGC CAGTA	
	92,819: GGTCA GTGCGCATGCGt CGGTA	
	92,857: GCGCC GTGCGCATGCGC CGGTA	
CGGTAWWWWWCCACT	92,757: TGCGC CGGTAAAATTCCACT GTGTG	92,935: GCGGC gGTGGAAAATTACCG CTCCG
(allowing 1 mismatch)	92,793: TGCGC CaGTATTTTTCCACT AGAGG	
	92,831: TGCGT CGGTAAAATTCCACT AGATG	
	92,869: TGCGC CGGTATTTTTCCACT GGGCG	
GGGRYCCCTCSCCC	93,128: GTCCG CGGGCCCgTCCCaCC GCCCT	93,458: CCCAC GGGGGGctGGGTCgC GGACC
(allowing 3 mismatches)	93,208: GGCCG GGGGTCCCTCCCCtC CCCCC	93,690: AGGTT GGGGGctGGGGgCCC GGTCA
	93,360: GGCCC GGGATCCCTCGCtgC TCCCG	
	93,512: GACCG GGGATCCCcCaCCCA GCTCC	
	93,782: CGGTT GGGCTCCCctCCCCC CTCTC	
	93,783: GGTTG GGctCCCCTCCCCCC TCTCG	
	93,828: GACCG GGGGTCCCTCGgCCT AGCCG	

[a] Coordinates are from the published nucleotide sequence, GenBank accession number X17403 (14). Mismatches are indicated with lowercase letters.

DISCUSSION

Results shown here define a segment of the HCMV AD169 genome, designated *ori*Lyt, that can direct initiation of DNA replication in transient transfection-plus-infection assays. This sequence lies near the center of the U_L component of the HCMV genome, upstream of UL57 (14). Clones containing the equivalent region of HCMV Towne also mediated DNA replication. Several lines of evidence argue that *ori*Lyt is an authentic origin for HCMV lytic-phase DNA replication and therefore provides a useful model for studying the molecular events initiating DNA replication and their regulation. First, *ori*Lyt-mediated replication, like lytic-phase virus genomic DNA replication, requires the virus-specified DNA polymerase activity. Presumably, other HCMV-contributed factors, including known homologs of the HSV replication genes such as the single-stranded DNA-binding protein (1), are also necessary, although this remains to be demonstrated. Second, restriction analysis of replication products provided evidence that *ori*Lyt-mediated DNA replication is "autonomous" in that replication apparently does not require recombination with the superinfecting virus genome. Third, partial digestion demonstrated that the products of *ori*Lyt-mediated DNA replication are tandem arrays, as products of virus lytic-phase genomic DNA replication are thought to be (12). These products are similar in structure to those generated by other herpesvirus lytic-phase amplicons (46, 50) but distinct from products of Epstein-Barr virus latent-phase DNA replication (24, 44, 55). Finally, *ori*Lyt lies in a region of the HCMV genome that recent experiments done by using a novel approach employing ganciclovir (25), a selective inhibitor of the viral DNA polymerase (7), suggested contain a replication origin. The precise site of initiation, if indeed there is a precise site, remains to be determined. Our finding that this region can direct DNA replication in the transient assay has allowed us to begin studies to define required elements within *ori*Lyt, as discussed below.

Several lines of evidence suggest that *ori*Lyt may be the only lytic-phase origin for HCMV DNA replication. First, various other cloned fragments were tested, including plasmid clones containing the major immediate early region and spanning the internal repeats as well as sets of overlapping cosmid clones comprising the entire genome (18); none besides those containing *ori*Lyt directed DNA replication in the transient assay (3). Second, preliminary deletion analysis, discussed below, suggests that one or more of the clustered repeated sequences unique to *ori*Lyt may be required for *ori*Lyt function. Finally, Hamzeh et al. (25) detected only a single amplified locus containing *ori*Lyt in DNA prepared from ganciclovir-treated HCMV-infected cells. Thus, *ori*Lyt must contain or lie near the primary site for initiation of genomic DNA replication in HF cells. Nevertheless, for several reasons, we cannot exclude the possibility that other replication origins exist. Critical elements may be lost spontaneously from cloned fragments (52). HCMV can replicate in several cell types (28, 31), and alternate sites of initiating replication may be used in nonfibroblast host cells. Finally, we note that, despite molecular archeology suggesting that HCMV does not replicate extensively during latency (29), it is possible that some latent-

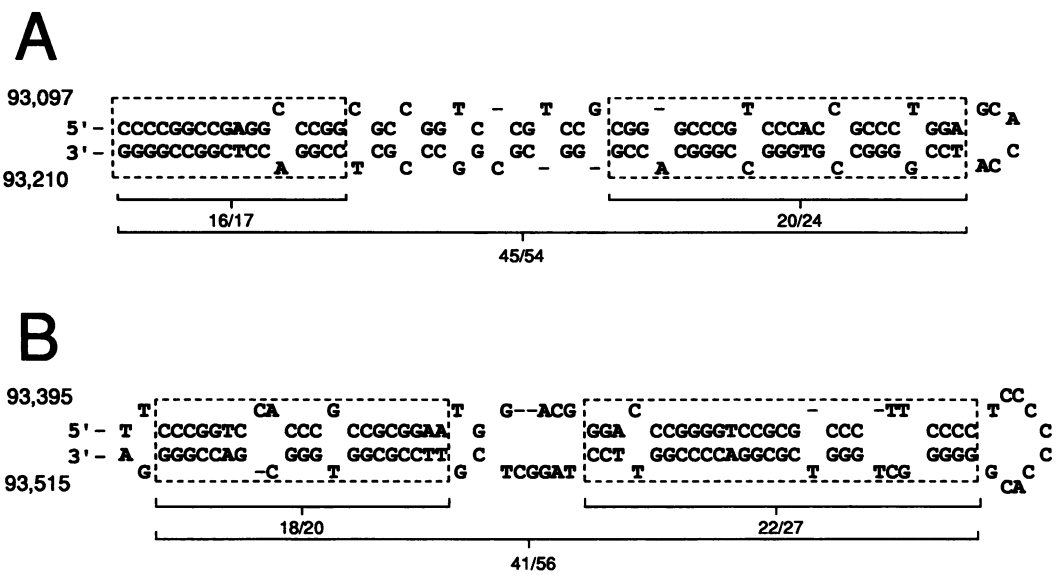

FIG. 7. Two large dyad symmetries. The nucleotide sequence containing *ori*Lyt was searched for dyad symmetries by using the GCG program STEMLOOP (16). The two largest, described in Results, are drawn here as hairpin loops solely to illustrate the extent of similarity between the arms. The fractions of matching nucleotides overall and within regions of the dyad arms are indicated.

phase DNA replication occurs under the direction of a separate origin.

The structure of HCMV *ori*Lyt. HCMV *ori*Lyt is remarkable among viral replication origins for its apparent size and complexity. Deletion analysis defined a 2.4-kbp core region containing sequences required for *ori*Lyt function lying between the *Aat*II site at nt 91,321 and the *Sac*I site at nt 93,715. However, additional elements flanking this core also clearly contributed to activity, because deletions around either boundary were progressively defective and because a construct combining the two independently defined boundaries failed to replicate in the transient assay. Deletion on the left to position 90,504 and on the right to position 93,930 had no detected effect on replication in the transient assay. Thus, by this measure, elements participating in *ori*Lyt function span 2.4 to 3.4 kbp.

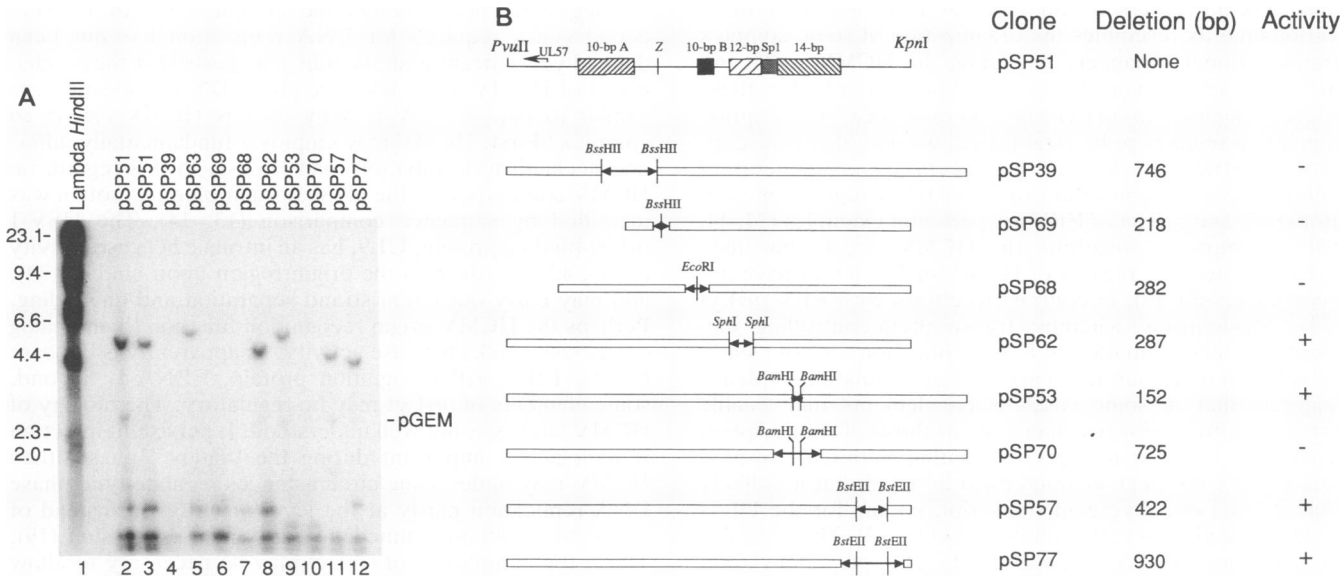

FIG. 8. Deletions within the boundaries of *ori*Lyt. (A) Transient transfection analysis. Transient replication assays were performed as described in Materials and Methods, and 1 μg of each sample DNA was cut with *Dpn*I plus *Eco*RI. In this experiment, an equimolar concentration of pGEM-7Zf(−) was included in each transfection as an internal negative control. Shown is a photograph of the resulting autoradiogram. Plasmid constructs used to generate the respective samples (discussed in the text) are indicated at the top. The positions of *Hin*dIII-cut lambda marker fragments (in kilodaltons) and linearized pGEM-7Zf(−) are indicated at the side. (B) Physical locations of internal deletions. The approximate positions of described deletions are diagrammed relative to the locations of various discussed sequence features present within *ori*Lyt. Precise deletion coordinates are given in the text.

Results obtained with constructs containing various deletions within and around these boundaries argue that multiple components within HCMV *ori*Lyt cooperate to promote and regulate initiation of lytic-phase DNA replication. Several repeated sequence motifs unique to this region of the HCMV genome, listed in Table 1, to our knowledge do not match known transcription factor or other protein-binding sites. These sites are obvious candidates for specific recognition by uncharacterized virus-coded or cellular factors or perhaps are novel sites recognized by known factors. One likely function for such a specific interaction is that of an origin recognition protein, although no homolog of the HSV-1 origin-binding protein (17, 42) was found in the HCMV genome (13, 14). Whatever their role, the clustering of these novel elements in *ori*Lyt suggests that they may form functional domains, or modules. Deleting segments containing any one of these *ori*Lyt-specific clusters, except for the 12- and 15-bp repeats (Fig. 8A, lane 8) (3), correlated with loss of origin function in the transient assay (e.g., pSP68, in which the 10-bp direct repeats were removed, and pSP70, in which the 14-bp repeat cluster was deleted). Other circumstantial evidence implicating some of the clustered elements in *ori*Lyt function is their apparent conservation in the cytomegalovirus Colburn lytic-phase replication origin (4). Nevertheless, we cannot yet conclude that these elements have a required role in origin function, because the tested deletions also removed other sequence information. More-detailed deletion and insertion mutagenesis as well as reconstruction analysis will be required to determine whether these elements are necessary.

Other reiterated elements within *ori*Lyt match binding sites of known transcription regulators, including ATF/CREB, MLTF/USF, and Sp1. These elements contribute to regulated expression from other HCMV promoters (e.g., see reference 33), and one or more of them may confer responsiveness to virus-coded immediate early *trans* activators (20). The interspersed and modular distribution of these various motifs resembles the organization of some complex transcriptional enhancers, including the HCMV major immediate early enhancer region. Though a role for these sequences either in *ori*Lyt function or in local transcription regulation remains to be established, we note that transcriptional control elements and enhancers are components of many eukaryotic and viral origins of DNA replication (15). Both *ori*P and *ori*Lyt of EBV are pertinent examples (24, 44, 56). Attempts to substitute the HCMV major immediate early enhancer for regions of HCMV *ori*Lyt so far have not been successful (3), in contrast to efforts with EBV *ori*Lyt (24). Mechanisms whereby transcription-controlling elements influence initiation of DNA replication are not clearly established and doubtless vary, but accumulating evidence suggests that in some cases, such elements may enable replication by pathways analogous to those of transcription activation (40). Transcripts originating within or around *ori*Lyt have not been characterized in detail, but it is likely that at least one divergent transcript, coding for the UL57 product, originates at the left of *ori*Lyt (3, 32). The presence of candidate TATA box, CCAAT, and polyadenylation signals in combination with the above-noted homologies to transcription-regulating elements raises the possibility that other transcripts arise and terminate within *ori*Lyt; preliminary results suggest that this is the case (3). It will be important to characterize these transcripts, as they may play a role in *ori*Lyt activity or regulation, or they may express proteins encoded by open reading frames present within the boundaries of oriLyt (14). In this regard, we note that transcripts have been found to traverse HSV-1 ori_s (30).

The asymmetric base composition within HCMV *ori*Lyt suggests two testable hypotheses. First, the A+T-rich segment between nt 91,100 and 91,300 may comprise a DNA-unwinding element, implying that initiation would occur within or near this segment. It has been demonstrated that many origins of replication include a DNA-unwinding element with intrinsic physical properties that provide a favored site for strand separation and, in conjunction with other elements, replication initiation (37, 49). This suggestion is consistent with results obtained by using the ganciclovir termination method that predicted initiation within a zone centered to the left of the *Eco*RI site at nt 92,210 (25), although that approach cannot precisely localize the initiation site. Deletion constructs spanning this region are impaired or inactivated in the transient replication assay. Similarly A+T-rich segments are present in the lytic-phase origins of SCMV Colburn and EBV. A second hypothesis is that the conserved G+C-rich segment contains a replication fork barrier. This region includes repeated homopyrimidine · homopurine tracts that might under certain conditions form triplex DNA structures (see reference 53 and references therein); such regions have been shown to impede the DNA polymerase and arrest DNA synthesis (6). To take this speculative hypothesis one step further, the currently favored rolling-circle model suggests that DNA replication may be oriented. If so, such a fork barrier might help establish orientation. It should be possible to use established methods to determine whether HCMV DNA replication is oriented.

Why is HCMV *ori*Lyt so complex? Described alphaherpesvirus replication origins have a comparatively simple structure, spanning around 100 bp and comprising a palindrome including a central A+T-rich region and recognition sites for an origin-binding protein (47, 48). The apparent complexity of the HCMV and EBV replication origins represents a striking difference. Though the complete set of HCMV-coded genes required for DNA replication has not been defined yet, current understanding suggests that the mechanisms of HCMV and EBV lytic-phase DNA replication are similar to that of HSV-1. Why then is HCMV *ori*Lyt so complex? First, HCMV may employ a fundamentally different mechanism of initiation than HSV-1. In this regard, no HCMV counterpart to the HSV-1 origin-binding protein was identified by sequence comparison (13, 14). The HSV-1 origin-binding protein, UL9, has an intrinsic helicase activity (9, 35, 42, 51), distorts the origin region upon binding (34), and may carry out initial strand separation and unwinding. Perhaps the HCMV origin recognition function (if an analog even exists) lacks helicase activity, as apparently is the case for the EBV *ori*P recognition protein, EBNA-1. Second, some elements of *ori*Lyt may be regulatory. The biology of HCMV latency is not well understood. If persistent infection is biologically important during the "latent" phase, then HCMV may under some circumstances regulate lytic-phase DNA replication partly at the level of initiation instead of solely by regulating immediate early gene expression (19). Third, the complexity of *ori*Lyt may be necessary to allow regulated replication in the wide variety of differentiated cell types thought to serve as permissive hosts in vivo (28). For example, if transcription factor binding plays a role in mediating replication initiation through any of the postulated mechanisms (15), then different subsets of elements may be utilized in different host environments. Fourth, if HCMV latent-phase DNA replication does occur, elements of *ori*Lyt

may be required; i.e., *ori*Lyt may contain or overlap a latent-phase origin of replication, in effect merging two functions performed by separate EBV loci. Finally, in light of the recent demonstration that flanking transcription control regions can augment HSV-1 ori_s activity (54), it is possible that HCMV *ori*Lyt is not fundamentally different but rather that the observed differences result from the lower relative activity of HCMV *ori*Lyt, which could make detection of basal function impossible. Whatever the answer, a full understanding of the functional roles of elements required for HCMV *ori*Lyt activity may provide the key that opens a door on important aspects of HCMV biology.

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