JOURNAL OF VIROLOGY, Feb. 1991, p. 931–937
0022-538X/91/020931-07$02.00/0

Vol. 65, No. 2

Multicomponent Origin of Cytomegalovirus Lytic-Phase DNA Replication

DAVID G. ANDERS* AND SUZANNE M. PUNTURIERI

Virology Laboratories, The Wadsworth Center for Laboratories and Research, State University of New York at Albany, School of Public Health, New York State Department of Health, Albany, New York 12201-0509

Received 22 August 1990/Accepted 5 November 1990

Cytomegalovirus (CMV) lytic-phase DNA replication requires both *trans*-acting factors, such as the virus-coded DNA polymerase, and a previously undefined *cis*-acting element, the origin, within which initiation occurs. We have located a candidate origin of CMV lytic-phase DNA replication, oriLyt, in both simian and human strains by assessing the ability of cloned restriction fragments to mediate phosphonoformic acid-sensitive DNA replication after transfection into human fibroblasts when required *trans*-acting factors were supplied by infection. In initial experiments the simian CMV-like strain Colburn *Eco*RI D fragment directed DNA replication; this fragment contains all of the single-stranded DNA-binding protein gene (*dbp*) and about 7 kbp of upstream sequence. A larger region upstream of human CMV *dbp* also mediated replication in transient assays. Subsequent subcloning and deletion analyses defined a CMV strain Colburn region sufficient for origin function, spanning about 1,300 bp in the apparently noncoding region upstream of *dbp*. The nucleotide sequence of this region revealed four distinct domains, containing (i) a 9-bp repeated sequence, (ii) an A+T-rich segment, (iii) an 11-bp direct repeat, and (iv) a 47-bp direct repeat. At least some part of each of these domains was required for origin function. Therefore, like the Epstein-Barr virus lytic-phase origin of DNA replication, CMV oriLyt appears to be structurally complex.

Human cytomegalovirus (HCMV) is a significant cause of morbidity and mortality after in utero or perinatal infection and among immunocompromised individuals (1). Because cytomegalovirus (CMV) exhibits both lytic and latent or persistent phases, like other herpesviruses, an understanding of the molecular mechanisms of CMV DNA replication and their regulation is needed to aid in developing effective antiviral strategies. Lytic-phase DNA replication requires both *trans*-acting factors, such as the virus-coded DNA polymerase (12, 18), and a previously unidentified *cis*-acting component that facilitates initiation, the origin of DNA replication. We used a transient transfection assay to locate a CMV lytic-phase replication origin, oriLyt, in both simian and human strains. Described herpesvirus replication origins vary in their structure and function; alphaherpesvirus (e.g., herpes simplex virus and varicella-zoster virus) replication origins consist of a single dyad symmetry, or a portion thereof, and a short flanking sequence (6, 24, 25), whereas both the latent-phase plasmid origin, oriP (21, 27), and the lytic-phase origin, oriLyt (10), of the gammaherpesvirus Epstein-Barr virus (EBV) are composed of multiple elements. Data presented here show that the betaherpesvirus CMV oriLyt is distinct from other herpesvirus replication origins, but that, like EBV oriLyt, it is structurally complex.

MATERIALS AND METHODS

Virus and cells. Human foreskin fibroblasts (HF cells), used for all experiments, were prepared, passaged, and infected as previously described (9). Cells used for transfection experiments were of a low to moderate passage number. CMV strains Colburn and Towne were obtained from Wade Gibson.

Recombinant plasmids. Plasmids DGA1 and DGA7, containing HCMV strain Towne *Bam*HI-K and *Hin*dIII-A, respectively, and DGA8, containing CMV strain Colburn *Eco*RI-D, have been described (3). The *Eco*RI-*Sma*I and *Eco*RI-*Bam*HI subclones of *Eco*RI-D drawn in Fig. 1d were constructed by treating pDGA8 with *Sma*I or *Bam*HI, excising the segment between the indicated restriction site within *Eco*RI-D and the adjacent corresponding site in the pUC18 polylinker, and religating to generate pDGA33 and pDGA34, respectively. The *Sal*I-*Sma*I and *Hin*dIII-*Sma*I subclones were made by treating pDGA33 with *Sal*I or *Hin*dIII, purifying the fragment delineated by the indicated restriction site within *Eco*RI-D and the adjacent vector polylinker site, and ligating the purified fragment into the corresponding site of pUC18, to generate pLD3 and pLD1, respectively. The *Hin*cII-1 subfragment of *Eco*RI-D was excised from pDGA8 and ligated into pUC18 in both a and b orientations to produce pSP16 and pSP17, respectively. *Xba*I-CF, containing fused *Xba*I-C and *Xba*I-F subfragments of *Eco*RI-D (3), was prepared by ligating gel-purified *Xba*I-F into the *Xba*I-C clone DGA10 after linearizing with *Xba*I. Independent *Hin*cII-*Sma*I subclones were constructed (i) by treating pSP16 with *Sma*I to excise the rightward half of the *Hin*cII-1 fragment and religating, generating pSP18, or (ii) by cutting pSP17 with *Sma*I, purifying the fragment flanked by the indicated *Eco*RI-D *Sma*I site and the *Sma*I site of the vector, and ligating into the *Sma*I site of pUC18 to give pSP19 or the *Sma*I site of pGem7zf(−) to give pSP21.

Transient transfection assay. For each transfection, about 1.0 pmol of plasmid DNA was mixed with 2 ml of serum-free Dulbecco's modified Eagle medium (DMEM)–50 mM Tris hydrochloride (pH 7.4) containing 400 μg of DEAE-dextran (Pharmacia; no. 17-0350-01) per ml and added to 6-cm dishes of HF cells split 1:4 24 h earlier that had been rinsed with Dulbecco's phosphate-buffered saline (D-PBS). After incubating at 37°C for 4 h, the transfecting mixture was removed, and the cells were treated with 2 ml of D-PBS containing

* Corresponding author.

10% (vol/vol) dimethyl sulfoxide for 1 min at room temperature. This mixture was aspirated, the dishes were washed once with D-PBS, and the cells were fed with DMEM containing 10% (vol/vol) fetal bovine serum. Transfected cells were infected with 10 to 100 PFU per cell 24 h later. DNA was prepared 72 h after CMV strain Colburn (SCMV) infection or 96 h after HCMV infection by adding to the D-PBS-washed cell pellet 500 μl of lysis buffer (5.0 mM Tris [pH 8.0], 100 mM EDTA, 400 mM NaCl, 0.5% [wt/vol] sodium dodecyl sulfate, 2.0% Sarkosyl) containing 200 μg of proteinase K per ml and incubating for 4 h at 37°C. The DNA was then purified by successive phenol and chloroform extractions and precipitated. Redissolved preparations were quantitated by fluorimetry, and 2 μg of each sample was incubated simultaneously with *Dpn*I and the specified second enzyme in a total volume of 200 μl. Restriction digests were checked for completeness by adding plasmid DNA to samples of the digestion or in parallel reactions to which plasmid DNA was added. Southern blot analysis of the resulting products was carried out as described previously (3).

DNA sequencing and analysis. The nucleotide sequence of the region containing SCMV oriLyt was determined by the dideoxy method with Sequenase (U.S. Biochemicals). Essentially complete sequences of both strands were obtained by bidirectional sequencing of various subclones and by filling in gaps with synthetic oligonucleotide primers where necessary. Sequence data were assembled and analyzed by using the GCG sequence analysis package (7).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned the accession number M57681.

RESULTS AND DISCUSSION

Location of SCMV oriLyt. Because previous attempts to find an HCMV origin of replication were unsuccessful, we tested selected cloned fragments from SCMV (9, 15) by using a transient assay. Briefly, cloned fragments to be tested were introduced into permissive HF cells by transfection, and the required *trans*-acting factors were supplied by SCMV infection. Then purified transfected-cell DNA was treated with the restriction enzyme *Dpn*I, which cleaves its 4-bp recognition sequence CATG only if the adenosine is methylated on both strands. Since mammalian cells do not normally methylate this position, replicated plasmid DNA is rendered *Dpn*I resistant; unreplicated input DNA remains *Dpn*I sensitive and is cleaved into small fragments. After DNA was treated with a second enzyme (*Bam*HI or *Sma*I in Fig. 1) to produce a unit fragment of known size, the replicated plasmid was distinguished from the input plasmid and from virus DNA by probing Southern transfers (22) with labeled vector DNA (pUC18).

In initial experiments the 12.1-kbp SCMV *Eco*RI D fragment (16) mediated DNA replication (Fig. 1a); *Eco*RI-D contains all of the single-stranded DNA-binding protein gene (*dbp*) plus about 7 kbp of 5′-flanking sequence (Fig. 1d) (2, 3). *Dpn*I-*Bam*HI digestion of DNA purified from HF cells transfected with an *Eco*RI-D-containing clone (pDGA8) yielded a 9.8-kbp pUC18-detected fragment (Fig. 1a, lane 4) that comigrated with the corresponding fragment produced by *Bam*HI digestion of pDGA8 (data not shown). pDGA8 consistently was amplified greater than fivefold, as estimated by comparing *Dpn*I-resistant and residual *Dpn*I-cleaved products by densitometry. In contrast, treating DNA purified from pUC18-transfected cells with *Dpn*I and *Bam*HI produced only *Dpn*I-cleaved fragments that were smaller than linearized 2.7-kbp pUC18 (Fig. 1a, lane 1). The background of *Dpn*I-resistant pUC18 observed in longer exposures was less than 1% of the *Dpn*I-resistant product detected from pDGA8-transfected cells (data not shown). As expected, there was no hybridization to identically prepared DNA from untransfected, infected cells (Fig. 1a, lane 2). Cells transfected with pDGA8, but not subsequently infected, failed to replicate the input plasmid (Fig. 1a, lane 3). Furthermore, treating pDGA8-transfected cells from the time of infection with phosphonoformic acid (13), a selective inhibitor of virus-encoded DNA polymerase, reduced plasmid replication about 10-fold, as estimated by densitometry (Fig. 1a, lane 5).

A formal possibility existed that *Dpn*I-resistant products obtained in the transient replication assays resulted from recombination into the superinfecting virus. To examine this possibility, DNA from a transient transfection assay of a replication-competent subclone of *Eco*RI-D (pSP19) was treated with *Dpn*I plus either *Apa*I, which does not cut pSP19 but does cut sites in the viral genomic DNA flanking the cloned segment (Fig. 1b, lane 1), or *Eco*RI, which cleaves pSP19 only once, within the vector (Fig. 1b, lane 2). Uncleaved *Dpn*I-resistant products detected by the vector probe were 20 kbp or larger; none corresponded to the predicted homologous recombination product, nor did any comigrate with supercoiled, open circlular, or linear plasmid DNA (Fig. 1b, lanes 3 [lower and upper bands] and 4, respectively). In other experiments, a significant fraction of uncleaved products failed to enter 0.8% agarose gels (data not shown). Cleaving only the unique vector site produced a single band that comigrated with linearized pSP19 (Fig. 1b, lanes 2 and 4). Similar results have been obtained with a variety of subclones and restriction enzymes. Such results are consistent with the production of tandemly repeated linear arrays of the input plasmid sequence, as predicted by the rolling-circle model. Most importantly, these observations together argue strongly against the possibility that replicated plasmid DNA resulted primarily from homologous recombination into the virus genome. We conclude therefore that SCMV *Eco*RI-D contains a sequence that can function as a replication origin, here designated oriLyt, in permissive cells and that its replication requires at least the virus-specified DNA polymerase. Other tested SCMV fragments failed to mediate DNA replication (data not shown). However, our data do not rule out the possibility that SCMV expresses other replication origins.

To further define SCMV oriLyt, we tested various subclones of *Eco*RI-D. Fragments containing the region upstream of *dbp* retained oriLyt activity. In the example shown (Fig. 1c), the 8.0-kbp *Eco*RI-*Sma*I subfragment (lane 3), the 4.0-kbp *Sal*I-*Sma*I subfragment (lane 4), and the 5.6-kbp *Hin*cII-1 subfragment in either a or b orientation (lanes 5 and 6, respectively) efficiently mediated replication, but replication of the 2.4-kbp *Xba*I-C–*Xba*I-F (3) (*Xba*I-CF; lane 7) subclone was not detected (Fig. 1d). The smallest tested subclone that supported DNA replication extended from the *Hin*cII site, 68 nucleotides (nt) upstream of the putative *dbp* translation start codon (2), about 2.2 kbp to the *Sma*I site (Fig. 1b). The location of SCMV oriLyt, immediately upstream of *dbp*, corresponds to that of herpes simplex virus ori_l.

Structure of SCMV oriLyt. The nucleotide sequence of this *Hin*cII-to-*Sma*I fragment (Fig. 2) showed SCMV oriLyt to be distinct from other herpesvirus replication origins and revealed four domains, each defined by a prominent struc-

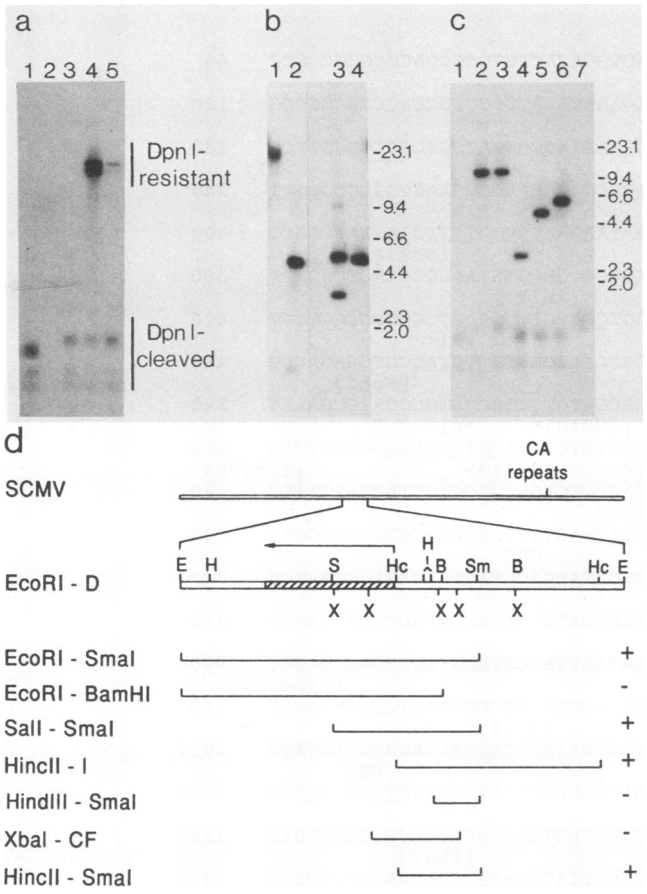

FIG. 1. Identification and location of CMV strain Colburn oriLyt. (a) Transient transfection assay showing *Eco*RI-D-directed DNA replication. HF cells were transfected (Materials and Methods) with pUC18 or with a clone (pDGA8) containing *Eco*RI-D and, unless otherwise noted, infected with CMV strain Colburn 24 h later. Transfected-cell DNA was digested with the restriction enzymes *Bam*HI and *Dpn*I, and about 2 μg of each sample was subjected to electrophoresis through a 0.8% agarose gel and transferred to a nitrocellulose membrane. The transfer was probed with ^{32}P-labeled pUC18, and the hybridizing fragments were visualized by autoradiography. Lanes: 1, pUC18-transfected cells; 2, untransfected cells; 3, pDGA8-transfected, uninfected cells; 4, pDGA8-transfected cells; 5, pDGA8-transfected cells treated with 200 μg of phosphonoformic acid per ml of medium from the time of infection. The second *Dpn*I-resistant band resulted from *Bam*HI star activity in the buffer used for this set of *Bam*HI-*Dpn*I double digestions. (b) Structure of *Dpn*I-resistant products. pSP19, containing the replication-competent *Hinc*II-to-*Sma*I subfragment of *Eco*RI-D (panel d), was assayed as described above, except for the following. A 0.5-μg sample of the purified transfected and infected-cell DNA was treated with *Dpn*I plus either *Apa*I, which does not cut pSP19 (lane 1), or *Eco*RI, which cleaves pSP19 once, in the pUC18 multiple cloning site (lane 2), and the samples were subjected to electrophoresis through a 0.5% agarose gel. *Apa*I-treated pSP19 (lane 3) and *Eco*RI-linearized pSP19 were analyzed in parallel for comparison; lanes 1 and 2 were exposed to film fivefold longer than were lanes 3 and 4. The positions and sizes (kilobase pairs) of *Hin*dIII-cut lambda fragments are given at the right. (c) Location of origin activity within *Eco*RI-D. The ability of the indicated cloned fragments to mediate replication was assayed as described above for panel a, except that 1 μg of each DNA sample was cut with *Sma*I and *Dpn*I. Cells were transfected with pUC18 (lane 1), with a clone containing *Eco*RI-D (lane 2) or clones containing the following *Eco*RI-D restriction subfragments (lanes): 3, *Eco*RI-*Sma*I; 4, *Sal*I-*Sma*I; 5, *Hinc*II-1 a orientation; 6, *Hinc*II-1 b orientation; 7, *Xba*I-CF. The positions and sizes (kilobase pairs) of *Hin*dIII-digested lambda fragments are indicated. Each result depicted in this figure has been repeated at least once. (d) Genomic location of the 12.1-kbp restriction fragment *Eco*RI-D and schematic summary of results localizing CMV (Colburn) oriLyt. The CA dinucleotide repeats define a position within the noninvertable SCMV genome roughly equivalent to the junction of short and long invertable segments of HCMV (16) and are indicated to show orientation. The position of *dbp* within *Eco*RI-D is indicated by cross-hatching; the 3.9-kb major *dbp* transcript is indicated by an arrow above. Restriction fragments used to construct subclones are drawn to scale underneath *Eco*RI-D. Fragment designations are given at the left, and activities in the transient replication assay are indicated to the right. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hc, *Hinc*II; S, *Sal*I; Sm, *Sma*I; X, *Xba*I.

tural motif. Domain I is situated proximal to the *dbp* promoter, between nt 200 and 650. It is characterized by multiple short tracts of T (or A) and by the presence of 11 copies (allowing one mismatch) of the 9-nt consensus 5′-CGGTG$(T)_4$/$(A)_4$ (R1) in either orientation. Domain II, extending from nt 650 to the *Xba*I site at nt 1185, does not appear to contain a prominent repeated motif but is A+T rich, with some 50-bp windows nearly 80% A+T, in contrast to surrounding sequences. Despite its high A+T content, domain II contains few T tracts. Also present in this region are three imperfect copies of the cyclic AMP-response element (20), two of which are adjacent, and nearby TATA consensus sequences. Domain III, flanked by the two *Xba*I sites, contains five direct repeats at roughly 50-nt intervals of the 11-nt consensus 5′-GCCCATCCCCC-3′ (DR2). This region also contains several short runs of T and one copy of R1. Domain IV, located between the *Xba*I site at nt 1642 and the *Sma*I site, is G+C rich and contains three adjacent copies of a 47-bp direct repeat (DR3), each exhibiting an internal dyad symmetry. Superimposed across the repeated 47-bp sequences is a pattern of alternating homopyrimidine and homopurine tracts. The arrangement of some of these features is summarized in Fig. 3d. In addition to the prominent motifs that define these domains, numerous other features are present. Among these are potential binding sites for known transcription-regulating factors, various inverted and direct repeats and, at the boundary between domains I and II, the sequence $(GC)_5$, which can potentiate the formation of Z-DNA (26).

SCMV oriLyt sequence requirements. To investigate the sequence requirements for origin function, we constructed nested sets of exonuclease III (ExoIII) deletions (14) from each end of the *Hinc*II-*Sma*I fragment, as well as several internal deletions, using convenient restriction sites, and we tested their ability to mediate DNA replication. The positions to which selected ExoIII deletions extended are given in Fig. 2. ExoIII deletions from the *Hinc*II site indicated that the minimal leftward boundary lies between nt 573 and 638. Deletions extending in as much as 345 nt from the *Hinc*II site did not reproducibly affect the ability of the resulting fragment to mediate DNA replication (Fig. 3a; pSP19*d*22). This deletion removes only 4 of 11 copies of R1 and only 10 of 24 T tracts. Deleting 573 nt from the *Hinc*II site, leaving only three copies of R1 and five T tracts, reproducibly reduced replication 5- to 20-fold, as estimated by densitometry (Fig. 3a, pSP19*d*101). Deleting the last three copies of R1 abolished the remaining replication (Fig. 3a; pSP19*d*108 and pSP19*d*114). Domain I is cleanly removed from pSP19*d*114, leaving domain II and the $(GC)_5$ sequence at its boundary intact. These results argue that a sequence(s) present be-

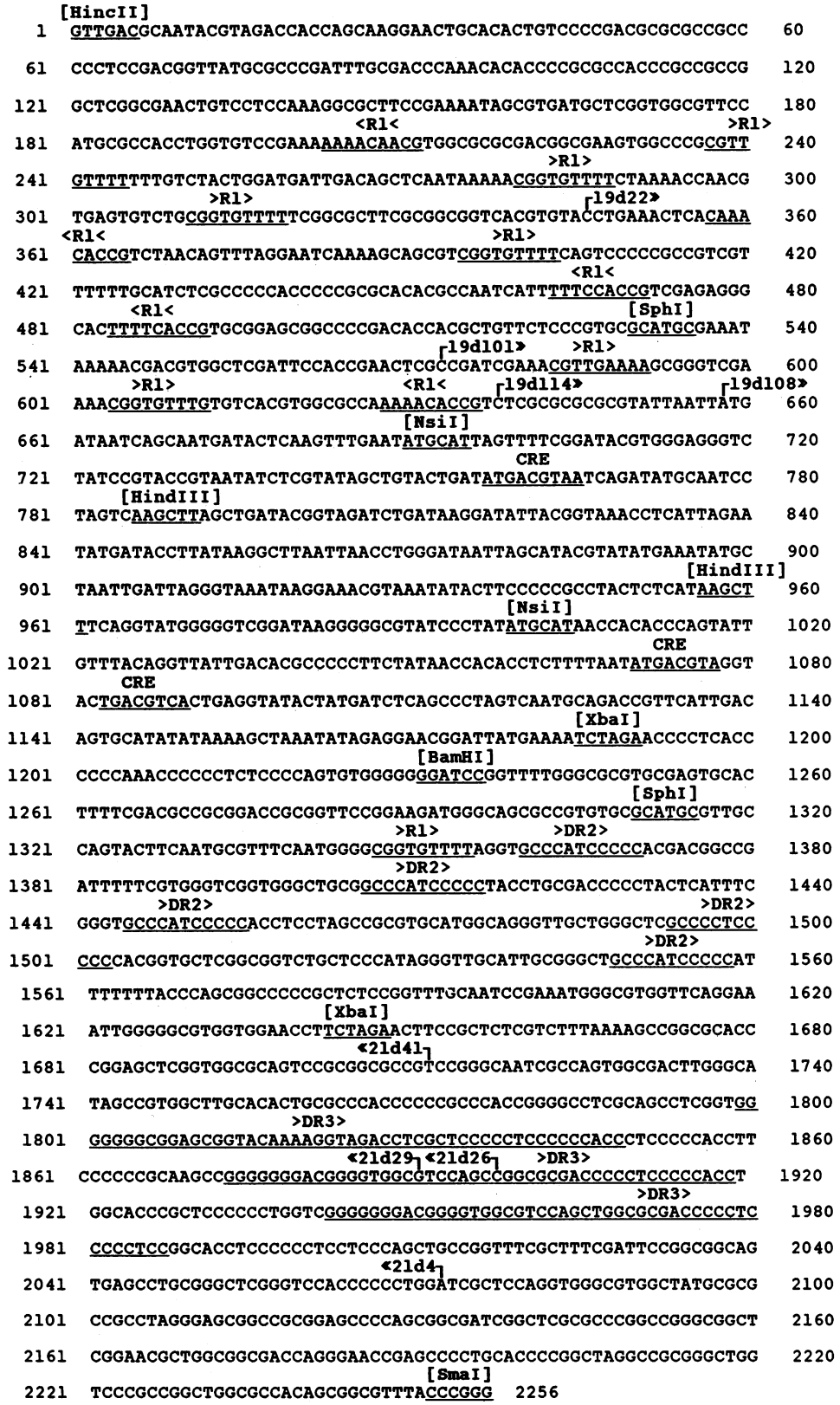

FIG. 2. Nucleotide sequence spanning SCMV oriLyt. Selected restriction sites and repeated sequences are underlined and denoted above the sequence. Numbering is from the *Hin*cII site. The boundaries of ExoIII deletions shown in Fig. 3 are indicated with an arrow above the sequence.

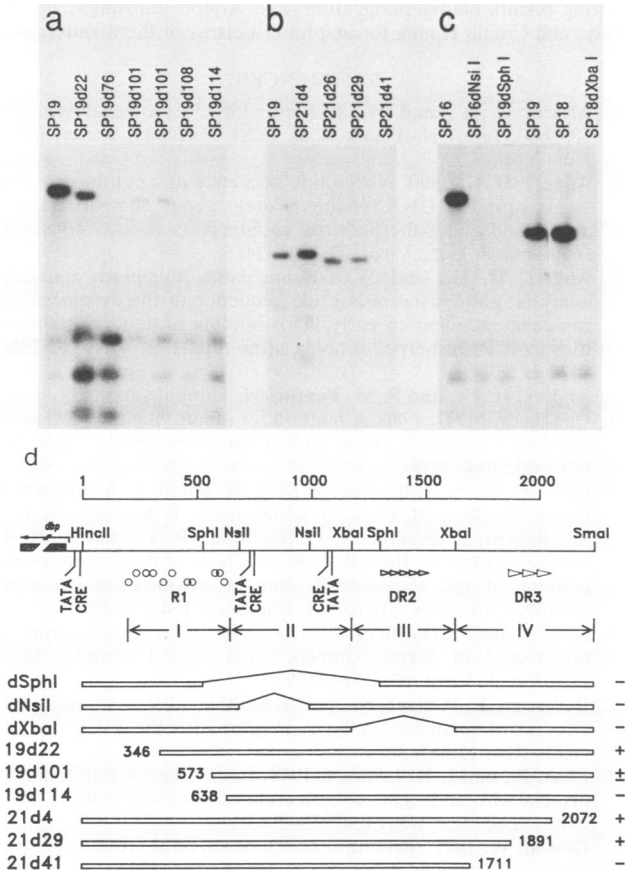

FIG. 3. Deletion analysis of SCMV oriLyt. (a) ExoIII deletions from the *Hinc*II restriction site. The clone pSP19, containing the *Hinc*II-*Sma*I subfragment of *Eco*RI-D inserted into the *Sma*I site of pUC18, was cleaved at the unique pUC18 *Pst*I site to protect the vector and then cut at the adjacent *Hinc*II site and treated with ExoIII for various times to produce a nested set of deletions, designated the pSP19*d* series. The extent of deletion was determined by sequencing selected clones (Fig. 2), and these clones were then tested for their ability to mediate DNA replication as described in the legend for Fig. 1. Results for representative deletions, indicated above the autoradiogram, are shown. (b) ExoIII deletions from the *Sma*I site. Clone pSP21, containing the *Hinc*II-*Sma*I fragment from SCMV *Eco*RI-D inserted into the *Sma*I site of pGEM7zf(−), was cut with *Apa*I to protect the vector and then at the adjacent unique *Eco*RI site and treated with ExoIII to produce the pSP21*d* deletion series. After sequencing to establish the extent of deletions, selected clones, indicated above the autoradiogram, were tested for their ability to mediate DNA replication as described in the legend to Fig. 1. (c) Internal deletions. pSP19*dNsi*I and pSP19*dSph*I were constructed by excising the internal 309-nt *Nsi*I and 881-nt *Sph*I fragments, respectively, from pSP19. The 457-nt *Xba*I fragment was excised from pSP18, containing the *Hinc*II-*Sma*I fragment inserted into the *Sma*I site of pUC18 in the orientation opposite to pSP19, to generate pSP18*dXba*I. The ability of the resulting fragments to mediate DNA replication was compared with those of controls as described above; transfected clones are indicated above the autoradiogram. pSP16 is the *Hinc*II-1 subfragment of SCMV *Eco*RI-D inserted into the *Hinc*II site of pUC18 in the a orientation. (d) Physical map of the *Hinc*II-*Sma*I restriction fragment, location of repeated sequence motifs, and summary of deletion analyses. The positions of the repeated sequence motif R1 are indicated with open circles; the positions of DR2 and DR3 are indicated with small arrows and with larger arrows, respectively, immediately below the physical map. Approximate boundaries of each domain, as described in the text, are also indicated. Locations of deletions are diagrammed relative to the physical map and motif summary; numbers indicate the deletion endpoints, as numbered in Fig. 2. Not indicated is the pSP19*d*76 deletion, which extended well beyond the junction with domain II.

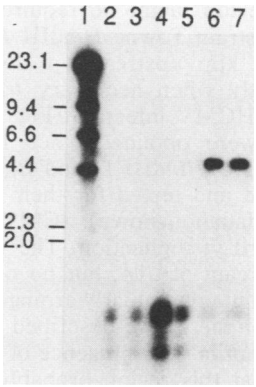

FIG. 4. Corresponding HCMV oriLyt. The ability of the plasmid clones pDGA1, containing HCMV strain Towne *Bam*HI-K (lanes 4 and 5), and pDGA7 (14), containing HCMV strain Towne *Hin*dIII-A (lanes 6 and 7), to mediate DNA replication in the assay described in the legend to Fig. 1 was compared with that of the vector control pUC18 (lanes 2 and 3). A 1-μg sample of each transfected-cell DNA preparation was digested with *Sma*I plus *Dpn*I. Lane 1 contains lambda phage DNA digested with *Hin*dIII, transferred, and probed in parallel as a marker.

tween nt 573 and 638, perhaps R1, is required for origin function. An alternative possibility is that removal of this segment brings elements of oriLyt adjacent to inhibitory vector sequences. ExoIII deletions from the *Sma*I site similarly suggested that the minimal rightward boundary lies between nt 1711 and 1891. Removal of up to 366 nt from the *Sma*I end, including one and one-half copies of DR3, had little if any effect on oriLyt function (Fig. 3b; pSP21*d*4, pSP21*d*26, and pSP21*d*29). However, removing an additional 179 bp, deleting the remaining 47-bp repeat plus about 80 bp, reduced oriLyt-mediated DNA replication to background level (Fig. 3b; pSP21*d*41), implying that DR3 and/or an adjacent sequence is required. Sequences needed for minimal oriLyt function therefore spanned about 1,300 bp, from nt 573 to 1890. Finally, deleting either of the internal domains also destroyed oriLyt function. Excising the 780-bp *Sph*I fragment between nt 530 and 1310, deleting all of the A+T-rich domain and part of domain I, abolished replication (Fig. 3c; pSP19*dSph*I). Deleting the 309-bp fragment between *Nsi*I sites at nt 690 and 999, removing about 60% of the A+T-rich region and one cyclic AMP response element consensus but leaving the tandem cyclic AMP response element sites and both ends of domain II intact, also inactivated oriLyt (Fig. 3c; pSP19*dNsi*I). Deleting the segment between the *Xba*I sites, removing all five copies of DR2, also eliminated origin activity (Fig. 3c; pSP18*dXba*I). Together, these preliminary deletion analyses (Fig. 3d) suggest that at least some portion of each domain is required for oriLyt function.

Corresponding HCMV oriLyt. In previous attempts to identify an HCMV origin of DNA replication, we tested the corresponding 7.8-kbp strain Towne *Bam*HI K fragment, which contains all of *dbp* and about 3 kbp of upstream sequence (3, 5, 17); it failed to replicate (Fig. 4, lanes 4 and 5). However, the size of SCMV oriLyt suggested that a yet

more extensive region might be required. Therefore, we tested the 23-kbp strain Towne *Hin*dIII A fragment, which extends about 17 kbp upstream of *dbp*. This fragment replicated efficiently when necessary *trans*-acting factors were supplied by HCMV infection (Fig. 4, lanes 6 and 7). Identical results were obtained when the corresponding HCMV strain AD169 *Hin*dIII D and *Bam*HI M (23) fragments were cloned and tested for their ability to mediate DNA replication (data not shown). SCMV infection failed to support HCMV oriLyt replication. The HCMV nucleotide sequence (5) upstream of *dbp*, but no other region of the HCMV genome, contains similarly arranged candidate counterparts to each of the above-described SCMV oriLyt repeated sequence motifs. The presence of these homologous motifs suggests that this region probably comprises all or part of an HCMV oriLyt. A primary difference between this region of HCMV and SCMV oriLyt is the absence of an A+T-rich region between candidate counterparts of domains I and III. Instead, an extended A+T-rich segment, not included in strain Towne *Bam*HI-K, is present further upstream of *dbp*. Hamzeh et al. (11) developed a novel method using the chain-terminating inhibitor ganciclovir [9-(1,3 dihydroxy-2-propoxymethyl)guanine] and obtained evidence that HCMV DNA replication initiates in vivo upstream of *dbp*. Moreover, they have now confirmed with their method that SCMV replication initiates in vivo within *Eco*RI-D (11a).

The results presented here, together with those of Hamzeh et al., clearly locate a region of the CMV genome that serves as an origin of lytic-phase DNA replication. The structure of this origin is distinct from those of other herpesviruses. Although functional roles of individual elements remain to be established, the presence of conserved repeated sequence motifs in domains I, III, and IV suggests that these domains may act, at least in part, by forming specific complexes with virus- or host-specified proteins. The observed divergence of repeated sequences between HCMV and SCMV might thus explain the failure of SCMV to support HCMV oriLyt replication. Domain I and domains III plus IV can independently augment transcription from the basal *dbp* promoter after infection (3a) and may function as transcription enhancers; enhancer elements are required components of several well-characterized virus replication origins, including both EBV oriP and EBV oriLyt (10, 21), but not of alphaherpesvirus replication origins. In addition, one or more of these motifs may serve as a binding site for an as yet unidentified origin recognition (8) or initiator protein. Intrinsic physical properties of portions of this region may also play a role in oriLyt function. For example, the A+T-rich domain II, which lacks a prominent repeated motif, may contribute a DNA-unwinding element (19). Our demonstration that this region can mediate DNA replication in transient transfection experiments will allow molecular dissection of CMV oriLyt and, together with the recently completed nucleotide sequence of HCMV strain AD169 (5), should facilitate finding the set of virus-coded proteins required for lytic-phase DNA replication (4) and lead to a clearer understanding of the molecular mechanisms of CMV DNA replication. Finally, the unique structural complexity of the CMV and EBV lytic-phase replication origins and their evident regulation during latency suggest they may be interesting models of mammalian origin function.

ACKNOWLEDGMENTS

We thank Wade Gibson for providing cells and virus strains; M. Chee for sequence data; Gary Hayward and Fayez Hamzeh for sharing results before publication; and Arlene Ramsingh, Jan Kiethly, and Cinnia Huang for helpful criticisms of the manuscript.

REFERENCES

1. **Alford, C. A., and W. J. Britt.** 1985. Cytomegalovirus, p. 629–660. *In* B. N. Fields et al. (ed.), Virology. Raven Press, New York.
2. **Anders, D. G.** 1990. Nucleotide sequence of a cytomegalovirus single-stranded DNA-binding protein gene: comparison with alpha- and gammaherpesvirus counterparts reveals conserved segments. J. Gen. Virol. **71**:2451–2456.
3. **Anders, D. G., and W. Gibson.** 1988. Location, transcript analysis, and partial nucleotide sequence of the cytomegalovirus gene encoding an early DNA-binding protein with similarities to ICP8 of herpes simplex virus type 1. J. Virol. **62**:1364–1372.

3a. **Anders, D. G., and S. M. Punturieri.** Unpublished data.

4. **Challberg, M. D.** 1986. A method for identifying the viral genes required for herpes DNA replication. Proc. Natl. Acad. Sci. USA **83**:9094–9098.
5. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchinson III, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell.** 1990. Analysis of the protein coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154**:125–169.
6. **Deb, S., and M. Doelberg.** 1988. A 67-base pair segment from the ori_s region of herpes simplex virus type 1 encodes origin function. J. Virol. **62**:2516–2519.
7. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
8. **Elias, P., and I. R. Lehman.** 1988. Interaction of origin binding protein with an origin of replication of herpes simplex 1. Proc. Natl. Acad. Sci. USA **85**:2959–2963.
9. **Gibson, W.** 1981. Structural and nonstructural proteins of strain Colburn cytomegalovirus. Virology **111**:516–537.
10. **Hammerschmidt, W., and B. Sugden.** 1988. Identification and characterization of oriLyt, a lytic origin of DNA replication of Epstein-Barr virus. Cell **55**:427–433.
11. **Hamzeh, F. M., P. S. Lietman, W. Gibson, and G. S. Hayward.** 1990. Identification of the lytic origin of DNA replication in human cytomegalovirus by a novel approach utilizing ganciclovir-induced chain termination. J. Virol. **64**:6184–6195.

11a. **Hayward, G.** Personal communication.

12. **Heilbronn, R., G. Jahn, A. Burkle, U.-K. Freese, B. Fleckenstein, and H. zurHausen.** 1987. Genomic localization, sequence analysis, and transcription of the putative human cytomegalovirus DNA polymerase gene. J. Virol. **61**:119–124.
13. **Helgstrand, E., B. Eriksson, N. G. Johansson, B. Lannero, A. Larsson, A. Misiorny, J. O. Noren, B. Sjoberg, K. Stenberg, G. Stenig, S. Stridh, B. Oberg, S. Alenius, and L. Philipsson.** 1978. Trisodium phosphonoformate, a new antiviral compound. Science **201**:819–821.
14. **Henikoff, S.** 1984. Unidirectional deletion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**:351–359.
15. **Huang, E.-S., B. Kilpatrick, A. Lakeman, and C. A. Alford, Jr.** 1978. Genetic analysis of a cytomegalovirus-like agent isolated from human brain. J. Virol. **26**:718–723.
16. **Jeang, K.-T. and G. S. Hayward.** 1983. A cytomegalovirus DNA sequence containing tracts of tandemly repeated CA dinucleotides hybridizes to highly repetitive dispersed elements in mammalian genomes. Mol. Cell. Biol. **3**:1389–1402.
17. **Kemble, G. W., A. L. McCormick, L. Pereira, and E. S. Mocarski.** 1987. A cytomegalovirus protein with properties of herpes simplex virus ICP8: partial purification of the polypeptide and map position of the gene. J. Virol. **61**:3143–3151.
18. **Kouzarides, T., A. T. Bankier, S. C. Satchwell, K. Weston, P. Tomlinson, and B. G. Barrell.** 1987. Sequence and transcription analysis of the human cytomegalovirus DNA polymerase gene. J. Virol. **61**:125–133.
19. **Kowalski, D., and M. J. Eddy.** 1989. The DNA unwinding

element: a novel, *cis*-acting component that facilitates opening of the *Escherichia coli* replication origin. EMBO J. **8**:4335–4344.

20. **Montminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodman.** 1986. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA **83**:6682–6686.
21. **Reisman, D., and B. Sugden.** 1986. *trans*-activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen-1. Mol. Cell. Biol. **6**:3838–3846.
22. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
23. **Spector, D. H., L. Hock, and J. C. Tamashiro.** 1982. Cleavage maps for human cytomegalovirus DNA strain AD169 for restriction endonucleases *Eco*RI, *Bgl*II, and *Hin*dIII. J. Virol. **42**:558–582.
24. **Stow, N. D., and A. J. Davison.** 1986. Identification of a varicella-zoster virus origin of DNA replication and its activation by herpes simplex virus type 1 gene products. J. Gen. Virol. **67**:1613–1623.
25. **Stow, N. D., and E. C. McMonagle.** 1983. Characterization of the TR_S/IR_S origin of DNA replication of herpes simplex type 1. Virology **130**:427–438.
26. **Wells, R. D.** 1988. Unusual DNA structures. J. Biol. Chem. **263**:1095–1098.
27. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A *cis*-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81**:3806–3810.