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Eleven Loci Encoding *trans*-Acting Factors Are Required for Transient Complementation of Human Cytomegalovirus *ori*Lyt-Dependent DNA Replication

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Recently we described the use of human cytomegalovirus (HCMV) cosmid clones in a cotransfection assay of HCMV *ori*Lyt replication (G. S. Pari, M. A. Kacica, and D. G. Anders, J. Virol. 67:2575–2582, 1993). We have now used this assay to identify 11 distinct required loci encoding *trans*-acting factors sufficient for transient complementation of *ori*Lyt-dependent DNA replication. This set includes all of the virus genes essential to initiate and perform DNA synthesis together with the virus genes required to express these replication functions from their native promoters. Six of the identified loci span open reading frames (ORFs) that encode homologs or probable homologs of herpes simplex virus type 1 replication genes, consistent with predictions based on sequence similarities and biochemical properties. These include the DNA polymerase UL54 and polymerase-associated protein UL44, the single-stranded-DNA-binding protein UL57, and proposed subunits of a helicase-primase complex, UL70, UL105, and UL101-102. Frameshift mutations in any one of these essential ORFs abrogated complementation of DNA replication. Three required loci, UL36-38, IRS1 (or TRS1), and IE1/IE2, encode known regulatory proteins. The remaining two loci span ORFs UL84 and UL112-113 and encode early temporal class nucleus-associated proteins of unknown function. Neither of these genes have been implicated previously in DNA replication or in regulating gene expression, nor have counterparts in herpes simplex virus type 1 or Epstein-Barr virus been described. The results presented here will facilitate investigation of the mechanisms and regulation of HCMV lytic-phase DNA replication.

After entering permissive cells and uncoating, the linear human cytomegalovirus (HCMV) genome is thought to circularize (42) and then to replicate in the nucleus by a mechanism producing "endless" concatemeric products that are subsequently cleaved and packaged during virion assembly (59). Although the overall scheme appears similar to that of other herpesviruses, the details of this process are not well established. HCMV contains one identified origin of lytic-phase DNA replication (*ori*Lyt), which directs in *cis* the initiation of DNA synthesis (5, 7, 34, 47). However, few of the proteins essential for *ori*Lyt-mediated replication have been defined, and our current models of HCMV DNA replication derive largely from comparison with the prototype herpes simplex virus type 1 (HSV-1).

Consistent with extensive genetic analyses by many laboratories (reviewed in reference 62), seven HSV-1 genes that are necessary and sufficient for DNA replication were identified by using a novel transient-transfection method (10, 11, 66). Candidate HCMV homologs of five of these HSV-1 replication genes were identified by sequence similarity and by biochemical studies (12); these encode a DNA polymerase (UL54) and a polymerase accessory protein (UL44) (24, 25), a single-stranded-DNA-binding protein (UL57) (3, 4, 6, 40), and predicted helicase and primase proteins (UL105 and UL70, respectively) (12, 46). In addition, open reading frames (ORFs) UL101 and UL102 were noted as positional counterparts of the essential HSV-1 genes UL9 and UL8, respectively (12). Nevertheless, with the exception of the DNA polymerase, there has been little evidence that these candidate homologs are actually components of the HCMV replication apparatus.

Traditional genetic approaches have proven difficult to apply to HCMV because of its inherent slow growth in culture (59) and because of the lack, until very recently (15), of an immortalized cell line that would allow the generation of viruses containing null mutations in essential genes. The inability to readily generate conditional mutants necessitates alternative approaches to identifying essential genes, such as those required for DNA replication. To provide a genetic test of the role of candidate HCMV homologs in viral DNA synthesis and as a means to identify other essential loci, we developed a complementation assay, based on the strategy devised by Challberg (10), using HCMV cosmids to supply *trans*-acting factors necessary to replicate cloned *ori*Lyt (50). In our assay, five cosmids are needed for complementation. Initial experiments confirmed that this assay provides a valid genetic test, and we used it to define essential loci in two of the required cosmids. The loci established in those experiments are UL44 and two known activators of gene expression, UL36-38 and IRS1, or its terminal repeat counterpart, TRS1. The major immediate-early (IE) region was also needed.

Here we present results locating the remaining genes essential for transient complementation of HCMV *ori*Lyt-dependent DNA replication. The complete set comprises 11 distinct loci. These experiments provide genetic evidence consistent with requirements for the previously identified candidate HCMV replication genes and in addition demonstrate requirements for UL84 and UL112-113, which have not previously been implicated either in HCMV DNA replication or in regulation of gene expression.

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MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblast cells were used for all experiments and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum (32). HCMV strain AD169 (ATCC VR-538) was maintained as frozen stocks. Infection was done at an approximate multiplicity of infection of 10. Nucleotide sequence coordinates are from the published DNA sequence data (12) (GenBank accession number X17403).

Plasmid constructs. The replication reporter pSP50, described previously (7), contains HCMV *ori*Lyt as a 5.6-kbp *Pvu*II-*Kpn*I fragment. pDGA33 contains CMV strain Colburn *ori*Lyt and the single-stranded-DNA-binding protein gene and has been described before (7). HCMV cosmid clones (30) were kindly supplied by A. Colberg-Poley with the permission of B. Fleckenstein. pSVH contains HCMV immediate-early regions 1 and 2 (IE1 and IE2) (58) and was a gift from R. Stenberg. pPSV, which contains ORFs UL112 and UL113 under the control of the HCMV IE enhancer-promoter (65), was a gift from D. Spector. pZP3 contains the ORF IRS1, and the construction of this clone was described previously (50). All subclones were made by excising fragments from a low-melting-point agarose gel and ligating them into pGEM7Zf(−) (Promega, Madison, Wis.) at the appropriate restriction endonuclease site within the polylinker region by standard procedures (53), as detailed below.

(i) Subclones of pCM1029. pZP34 contains the 20.3-kbp *Hin*dIII F fragment, pZP35 contains the 22.8-kbp *Hin*dIII D fragment, and pZP27 contains the 7.3-kbp *Eco*RI fragment M extending from nucleotides (nt) 74117 to 81435. pZP33 was made by cleaving pZP27 with *Sac*I and *Eco*RI, filling in the resulting *Sac*I-*Eco*RI fragment extending from nt 76642 to 81435 with Klenow fragment, and ligating it into the pGEM7Zf(−) *Sma*I site to make pZP33NA. The UL54 ORF was then excised from pZP33NA by treatment with *Hin*dIII and *Xba*I, and the resulting 4.8-kbp fragment was ligated into pRC/RSV (Invitrogen) in the correct orientation to supply a polyadenylation signal for UL54, making pZP33. pZP37 contains the 14.3-kbp *Sph*I fragment extending from nt 78262 to 92576. pZP28 was made by ligating the 12.6-kbp *Sph*I-*Dra*I fragment extending from nt 78262 to 90842 into *Sph*I- and *Sma*I-treated pGEM7Zf(−). pΔZP28 then was made by cleaving pZP28 with *Bsi*CI, removing the segment extending from nt 82228 to 85965, filling in the *Bsi*CI ends with Klenow fragment, and religating. This manipulation is predicted to remove all but 14 codons of ORF UL56 as well as 421 amino-terminal codons from UL55 (a homolog of HSV-1 gB), giving a product with 484 amino acids of the carboxyl-terminal half. pZP30 contains the 14.6-kbp *Bam*HI fragment extending from nt 93513 to 108088. pZP29 was made by cleaving pZP30 with *Kpn*I and *Stu*I and ligating the resulting 4.1-kbp fragment extending from nt 99700 to 103827 into *Kpn*I- and *Sma*I-treated pGEM7Zf(−). A frameshift mutation in UL54 was made by cleaving pZP33 with *Age*I at nt 79414, filling in with Klenow fragment, and religating to make pZP33FR. A UL57 frameshift was made by cleaving pΔZP28 with *Asc*I at nt 89039, filling in with Klenow fragment, and religating to produce pΔZP28FR. A frameshift in UL70 was constructed by cleaving pZP29 with *Dra*II at nt 103013, filling in with Klenow fragment, and religating to make pZP29FR.

(ii) Subclones of pCM1039. pZP9 contains the 15.9-kbp *Eco*RI D fragment, pZP10 contains the 12.5-kbp *Xba*I fragment extending from nt 117485 to 130010, pZP31 contains the 11.2-kbp *Kpn*I fragment extending from nt 133232 to 144435, and pZP32 contains the 16.6-kbp *Xba*I fragment extending from nt 130010 to 146605. pZP11 was made by excising from pZP10 the 10.2-kbp *Nsi*I-*Eco*RI fragment extending from nt 119499 to 129744 and ligating into the corresponding pGEM7Zf(−) sites. pZP12 was made by subcloning from pZP11 the 5.2-kbp *Aat*II fragment extending from nt 120502 to 125708. pZP13 was made by subcloning from pZP12 the 2.9-kbp *Aat*II-*Fsp*I fragment extending from nt 120502 to 123363 and ligating it into *Aat*II- and *Sma*I-treated pGEM7Zf(−). pZP14 was made by subcloning from pZP10 the 4.6-kbp *Sac*I fragment extending from nt 122947 to 127507. A frameshift mutation in UL84 was made by cleaving pZP13 with *Mun*I at nt 122665, filling in with Klenow fragment, and religating to make pZP13FR.

(iii) Subclones of pCM1058. pZP15 contains the 13.1-kbp *Hin*dIII-*Eco*RI fragment extending from nt 142993 to 156125. pZP16 was made by subcloning from pZP15 the 8.0-kbp *Sac*I fragment extending from nt 143128 to 151125. pZP17 was made by cleaving pZP16 with *Sma*I at nt 144928 and in the polylinker region of pGEM7Zf(−), deleting the intervening 1.8 kbp, and religating. pZP18 was made by cleaving pZP16 with *Sph*I at nt 146036 and in the polylinker region of pGEM7Zf(−) to delete the intervening 2.9-kbp fragment and religating. pZP19 was made by subcloning from pZP18 the 3.6-kbp *Sph*I-*Hin*dIII fragment extending from nt 146103 to 149645. pZP39 was made by cleaving pZP19 with *Sph*I and *Asc*I at nt 146036 and 146384, respectively, removing the resulting 348-bp fragment, filling in the ends with Klenow fragment, and religating. pZP21 contains the 6.2-kbp *Hin*dIII T fragment. pZP20 was made by excising from pZP21 the 4.8-kbp *Stu*I-*Sma*I fragment extending from nt 150312 to 155121 and ligating it into the pGEM7Zf(−) *Sma*I site.

pZP38 contains the 20-kbp *Hin*dIII fragment E. pZP22 was made by cloning from pCM1058 the 9.1-kbp *Hin*dII-*Kpn*I fragment extending from nt 156251 to 165326. pZP26 contains the 11.5-kbp *Kpn*I-*Hin*dIII fragment extending from nt 165326 to 176844 ligated into the corresponding pGEM7Zf(−) sites. pZP23 was made by subcloning from pZP22 into pGEM7Zf(−) the 4.4-kbp *Sph*I-*Sac*I fragment extending from nt 159669 to 164152. pZP24 was made by cleaving pZP23 with *Msc*I at nt 163028 and 163950, removing the 922-bp fragment, and religating. pZP25 was made by subcloning from pZP23 the 1.8-kbp *Eco*RI fragment extending from nt 162053 to 163835. A frameshift mutation in UL102 was made by cleaving pZP19 with *Kpn*I at nt 147618, filling in with Klenow fragment, and religating to make pZP19FR. UL105 was frameshifted by treating pZP20 with *Age*I at nt 152349, filling in with Klenow fragment, and religating to make pZP20FR.

Transfection and replication assay. The modified $CaPO_4$ transfection procedure (13) and the assay for detection of replicated products were performed as described previously (50). Transfections were performed in duplicate and done at least twice. Total-cell DNA extracted from transfected cells was treated with 10 U of *Dpn*I and 25 U of *Eco*RI in a volume of 50 μl at 37°C for 4 to 16 h, subjected to electrophoresis through a 0.8% agarose gel, and transferred to a Zeta-probe nylon membrane (Bio-Rad, Richmond, Calif.) by the alkaline transfer method according to the manufacturer's instructions. Hybridizations were carried out with a ^{32}P-labeled random primer-generated pGEM7Zf(−) probe with an approximate specific activity of 10^9 cpm/μg. Filters were hybridized with 5 ng of probe in 10 ml of hybridization buffer (1.5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA (pH 7.7)], 7% [wt/vol] sodium dodecyl sulfate [SDS], 10% [wt/vol] polyethylene glycol) for 16 h at 68°C in a hybridization oven (Robbins Scientific). Posthybridization washes were performed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium

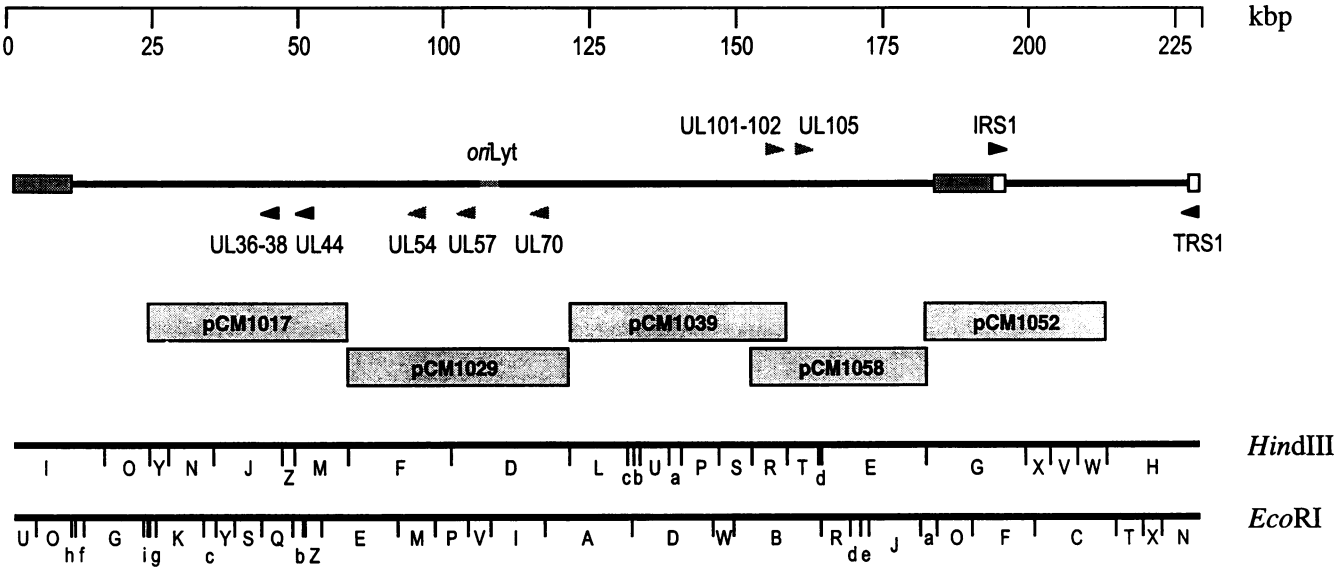

FIG. 1. *Hin*dIII and *Eco*RI physical maps of HCMV AD169 (12, 49), and HCMV cosmid clones used to establish the cotransfection complementation assay for *ori*Lyt-dependent DNA replication. Drawn to scale is a representation of the HCMV genome, indicating as landmarks the positions of the inverted repeats and *ori*Lyt. Indicated above and below the genome are the positions and orientations of candidate HCMV replication gene homologs (shaded arrowheads) and ORFs previously shown to be required for transient complementation of DNA replication (solid arrowheads) (50). The ruler (top) indicates genomic position in 25-kbp units.

citrate)–0.1% SDS twice for 15 min each at room temperature and then with 0.1× SSC–0.1% SDS twice for 45 min each at 68°C. Southern blots were then exposed to X-Omat AR (Kodak) X-ray film at −80°C for 24 h.

RESULTS

UL54, UL57, and UL70 are required for complementation of origin-dependent DNA replication. In previous experiments establishing the transient complementation assay, we demonstrated that five cosmids could express all of the functions necessary for DNA replication (50) and identified the essential loci in two of the required cosmids. One of the remaining three uncharacterized cosmids, pCM1029 (Fig. 1), spans three ORFs that were previously identified as likely homologs of HSV-1 DNA replication genes: the HCMV DNA polymerase (UL54), the single-stranded-DNA-binding protein (UL57), and one of the subunits for the helicase-primase complex (UL70) (12). To test whether these ORFs are indeed needed to complement DNA synthesis, as predicted from their similarity to HSV-1 replication functions, and to determine whether this region contains other essential genes, we used the published sequence of HCMV as a guide to construct subclones from pCM1029 so that they could be individually omitted. Each of the cotransfections for this series of experiments contained the indicated subclones of pCM1029 plus pCM1017, pCM1039, pCM1058, pZP3, pSVH, and the replication reporter plasmid pSP50.

Initially we found that pCM1029 could be replaced with three plasmid subclones, ZP34, ZP37, and ZP30 (Fig. 2A), each spanning the predicted transcript for one of the candidates noted above. Subclones of each of these plasmids then were tested to establish the essential ORFs (Fig. 2B, C, and D). pZP34, which spans ORFs UL48 to UL55 (Fig. 2A), including the DNA polymerase, replaced pCM1029 when subclones pZP28 and pZP30 were also included in the transfection cocktail (Fig. 2B, lanes 3 and 4). pZP27, which contains only ORFs UL53 and UL54, also complemented replication, indicating that all other ORFs present in pZP34 were dispensable (Fig. 2B, lanes 7 and 8).

The transcript reported to express the DNA polymerase (UL54) is about 5 kb in size (41), and a candidate polyadenylation signal is located within the UL53 coding sequence. To confirm that UL54 is the only pZP34 ORF required and to ensure efficient expression of the UL54 gene product, we eliminated UL53 and replaced the deleted polyadenylation signal by ligating the UL54 ORF, along with its promoter, into the expression vector pRc/RSV to make pZP33. pZP33 efficiently replaced subclone pZP27 (Fig. 2B, lanes 9 and 10). In this regard, we also note that a subclone containing only the UL54 ORF and its promoter, with no polyadenylation signal, failed to complement replication (51). Finally, when UL54-expressing plasmids were omitted from the cotransfection (e.g., Fig. 2B, lanes 5 and 6), or when UL54 was replaced with the UL54 frameshift mutant pZP33FR (Fig. 2B, lane 11), no replication was detected.

The transcript encoding the single-stranded-DNA-binding protein UL57 is 10 to 12 kb long (40) and continues far downstream of UL57 (51), which extends from nt 90326 to 86577. Consistent with these observations, *Hin*dIII-D (pZP35), which contains the complete UL57 ORF and its promoter but only limited downstream sequence, did not complement replication (Fig. 2C, lanes 3 and 4). Therefore, we started with a subclone containing the 14.3-kbp *Sph*I fragment extending from nt 78262 to 92576 (pZP37; Fig. 2A) and spanning the entire predicted UL57 transcription unit; this clone complemented replication (Fig. 2C, lanes 1 and 2). Subclone pZP28, which retains ORFs UL55 through UL57 but eliminates UL58 and UL59, was the smallest undeleted subclone supplying UL57 that we tested which complemented replication (Fig. 2C, lanes 5 and 6).

We note that all of the essential components of *ori*Lyt are deleted in pZP28 (5). To determine whether UL55 and UL56 are required, we made deletion constructs. Subclone pΔZP28, which removes from the predicted polypeptide sequences all

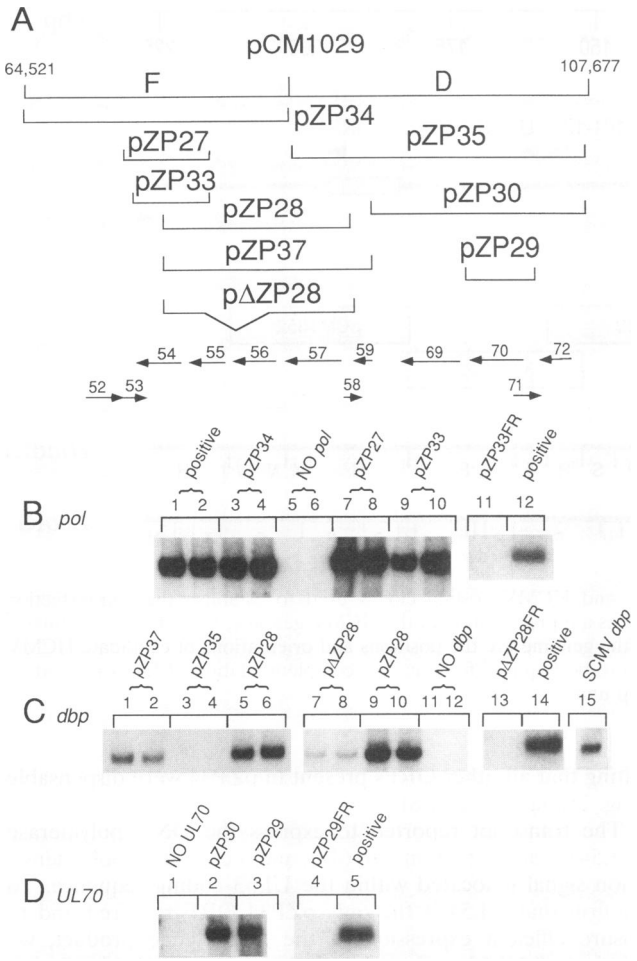

FIG. 2. Cosmid pCM1029 contains three loci required for complementation of *ori*Lyt-dependent DNA replication. (A) Physical map depicting *Hin*dIII fragments within cosmid pCM1029, the positions of other fragments that were subcloned and used in cotransfections, and the locations of predicted ORFs. Transient-complementation experiments were carried out as detailed in the text. Southern transfers of the *Dpn*I-treated transfected-cell DNA were exposed to film for about 24 h at −80°C. Presented here are the resulting autoradiograms; only the region in which *Dpn*I-resistant products are located is shown. All positive controls for these experiments included pCM1029, pCM1017, pCM1039, pCM1058, pZP3, pSVH, and pSP50 in the cotransfection cocktail. (B) Evidence that the HCMV DNA polymerase UL54 is required for complementation of *ori*Lyt replication. Cotransfection cocktails for lanes 3 to 11 were identical to the positive controls except that cosmid pCM1029 was replaced by pZP28 and pZP30 plus the plasmid indicated above each lane. (C) Evidence that the HCMV single-stranded-DNA-binding protein UL57 is required for complementation of *ori*Lyt replication. Cotransfection cocktails for lanes 1 to 13 and lane 15 were identical to the positive controls except that cosmid pCM1029 was replaced by pZP30 and pZP27 plus the plasmid indicated above each lane. (D) Evidence that the putative HCMV primase activity UL70 is required for complementation of *ori*Lyt replication. Cotransfection cocktails for lanes 1 to 4 were identical to the positive control except that cosmid pCM1029 was replaced by pZP27 and pZP28 plus the plasmid indicated above each lane.

but 14 codons of the ORF UL56 and 421 amino-terminal codons of UL55, complemented DNA replication, albeit at a reduced level (Fig. 2C, lanes 7 and 8), arguing that UL57 is the only ORF required from pZP28. Further evidence that UL57 is the only pZP28 gene required is that a clone expressing the CMV strain Colburn single-stranded-DNA-binding protein (pDGA33 [7]) can replace HCMV pZP28 in the replication assay (Fig. 2C, lane 15). Finally, when UL57 was omitted (Fig. 2C, lanes 11 and 12) or replaced with a clone containing a frameshift mutation in UL57 (pΔZP28FR), no replication was detected (Fig. 2C, lane 13).

The third proposed replication gene homolog within pCM1029 is HCMV UL70, supplied in initial experiments by pZP30 (Fig. 2A). To test whether UL70 was sufficient to replace pZP30, we made a subclone (pZP29) which contains only UL70 and found that it replaced pZP30 in the complementation assay (Fig. 2D, lane 3). Cotransfection cocktails omitting clones containing UL70 produced no signal (Fig. 2D, lane 1), nor did cocktails substituting a frameshift mutant of UL70 (Fig. 2D, lane 4). Together, these results indicate that UL70 is essential for complementation of DNA replication.

We concluded from this series of experiments that three subclones, pZP33, pΔZP28, and pZP29, which contained UL54, UL57, and UL70, respectively, are necessary to complement DNA replication and that these three alone are sufficient to replace cosmid pCM1029 in the replication assay.

pCM1039 contributes ORF UL84. Cosmid pCM1039 was also required for origin-dependent DNA replication (Fig. 1). This cosmid extends from nt 107677 to 149645 and includes *Hin*dIII fragments L, c, b, U, a, P, S, and R (Fig. 3A). ORFs UL101 and UL102, which were noted as positional counterparts of HSV-1 replication genes UL9 and UL8, respectively (12), are present both in this cosmid and in another required cosmid, pCM1058. However, a subclone of pCM1039 containing the UL101-102 region alone failed to replace pCM1039 in complementing replication (51). Therefore, we constructed a series of pCM1039 subclones (Fig. 3A) and determined which ones could replace pCM1039. Transfection cocktails for these experiments included cosmids CM1017, CM1029, CM1058, pZP3, pSVH, and pSP50 plus either pCM1039 (positive) or the indicated substitute(s) for pCM1039 (Fig. 3B and C). Cotransfecting either pZP32 plus pZP9 or pZP31 plus pZP9 in place of pCM1039 produced a positive replication signal (Fig. 3B, lanes 5 to 8). pZP31 plus pZP32 failed to replace pCM1039 (Fig. 3B, lanes 3 and 4). However, either pZP9, which spans ORFs UL84 to UL92, or pZP10, which spans UL82 to UL86, substituted for cosmid pCM1039 in the replication assay in the absence of pZP31 and pZP32 (Fig. 3B, lanes 9 to 12), defining an essential region spanning UL83 to UL86. Subsequently, we found that pZP11, which includes ORFs UL84 to UL86, also complemented replication (Fig. 3C, lanes 11 and 12), whereas pZP14, which spans ORFs UL85 and UL86, failed to complement (Fig. 3C, lanes 3 and 4). Another subclone, containing only UL84 and UL85 (pZP12), was also sufficient to replace pCM1039 (Fig. 3C, lanes 5 and 6). Finally, a subclone that contained only UL84 (pZP13) complemented replication whether alone or in combination with a clone spanning UL85 (pZP14; Fig. 3C, lanes 7 and 8). Thus, UL84 alone was sufficient to replace pCM1039 in the complementation assay (Fig. 3C, lanes 9 and 10). Omitting UL84 from transfections (Fig. 3C, lanes 1 and 2) or replacing it with a clone containing a frameshift mutation in UL84 (Fig. 3C, lane 15) abrogated complementation.

pCM1058 contributes four loci essential for transient complementation of DNA synthesis. The last required cosmid that we characterized, pCM1058, includes UL105, a candidate homolog of the essential HSV-1 DNA helicase UL5 (12, 46); ORFs UL101 and UL102, which were noted to be "positional" counterparts of HSV-1 replication genes UL9 and UL8, respectively; and the major immediate-early region (Fig. 1).

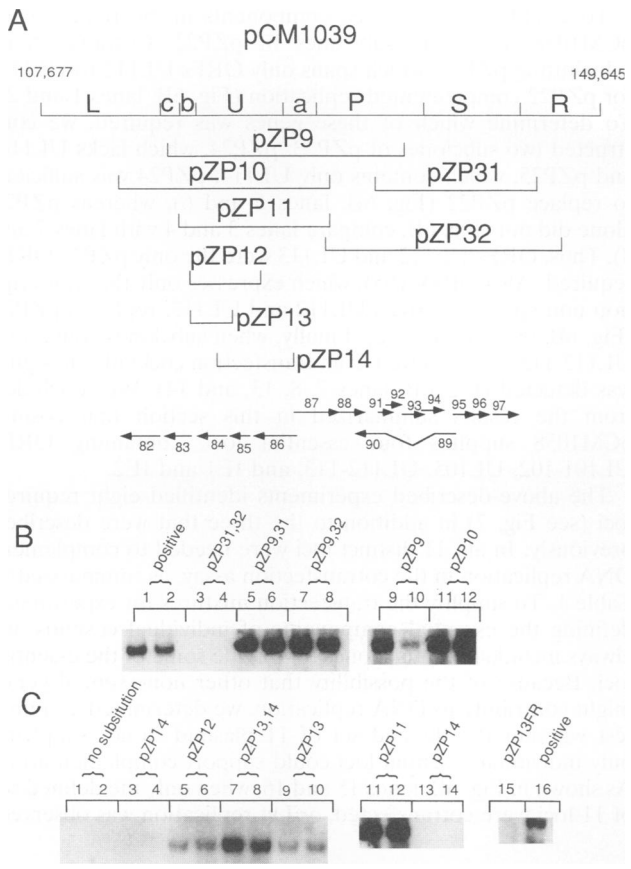

FIG. 3. Cosmid pCM1039 contributes UL84. (A) Physical map depicting *Hin*dIII fragments within cosmid pCM1039 and other subcloned fragments that were used in cotransfection experiments detailed in the text. Also shown are the predicted ORFs located in this region. (B and C) Transient-complementation experiments were done as described in the legend to Fig. 2. All positive controls (B, lanes 1 and 2; C, lane 16) for these experiments included pCM1029, pCM1017, pCM1039, pCM1058, pZP3, pSVH, and pSP50 in the cotransfection cocktail. Presented are photographs of the *Dpn*I-resistant portion of the Southern transfers. Cotransfections for all lanes contained the substitution(s) for pCM1039 indicated above the lanes plus pCM1017, pCM1029, pCM1058, pZP3, pSP50, and pSVH.

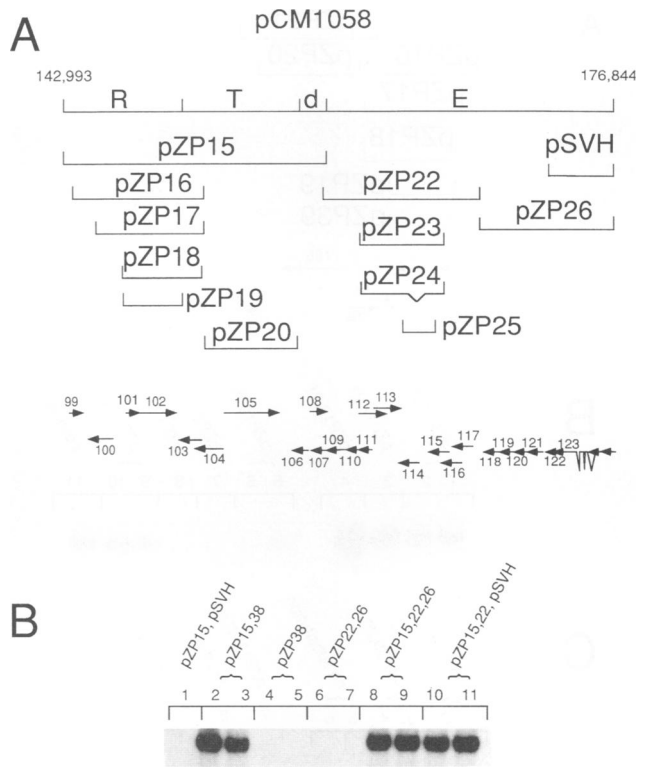

FIG. 4. Cosmid pCM1058 contains multiple essential loci. (A) Physical map depicting *Hin*dIII fragments within cosmid pCM1058 and fragments that were subcloned and used in cotransfections. Also indicated are the ORFs located within this region. (B) Autoradiogram of a Southern transfer showing *Dpn*I-resistant *ori*Lyt-containing products from cotransfection experiments. Transfections were carried out as described in Materials and Methods. All transfection cocktails contained pCM1017, pCM1029, pZP13, pZP3, and pSP50 plus either pCM1058 or the plasmids indicated above the lanes in place of pCM1058.

Therefore, we first attempted to replace pCM1058 by cotransfecting a large subclone, pZP15, encoding ORFs UL99 to UL106 (Fig. 4A) plus pSVH; this combination failed to complement replication (Fig. 4B, lane 1). Subsequently, we replaced pCM1058 with two plasmids, pZP15 and a subclone containing the *Hin*dIII E fragment (pZP38). This combination complemented DNA replication (Fig. 4B, lanes 2 and 3), suggesting that a second *Hin*dIII-E locus, in addition to the major immediate-early region, was needed. Therefore, we constructed additional subclones of *Hin*dIII-E (Fig. 4A). Cotransfections which included subclones pZP15, pZP22, and pZP26 in place of cosmid pCM1058 yielded replication (Fig. 4B, lanes 8 and 9). Moreover, pSVH replaced pZP26, showing that no pZP26 ORFs outside of the major immediate-early region are needed to complement DNA replication (Fig. 4B, lanes 10 and 11) and that pZP22, comprising ORFs UL110 to UL117 (Fig. 4A), contains a distinct essential function (Fig. 4B, compare lane 1 with lanes 10 and 11).

To establish essential loci in the left half of pCM1058, we constructed subclones of pZP15 containing ORFs UL100 to UL103 (pZP16) and UL105 and 106 (pZP21). These constructs together, in cotransfection experiments that also included pZP22 and pSVH, replaced pCM1058 (Fig. 5B, lanes 1 and 2). Smaller subclones of pZP16 (pZP17 and pZP18; Fig. 5A) ruled out the possibility that UL100 is needed for replication (Fig. 5B, lanes 3 and 4). The construct pZP19, lacking the ORF UL103, still complemented replication (Fig. 5B, lanes 5 and 6). Finally pZP39, which extends only 7 nt upstream from the putative ATG of UL101 and about 470 nt 3′ with respect to the putative translational stop of UL102, also complemented replication (Fig. 5C, lanes 3 and 4), arguing that not all of UL101 may be required for replication because any upstream promoter region needed for UL101 expression is missing. However, a construct in which most of the UL101 ORF was deleted failed to complement replication (51). pZP20, which contains only UL105, could replace pZP21 (Fig. 5B, lanes 9 and 10). Transfection mixtures omitting either UL101-102 (Fig. 5B, lane 7) or UL105 (Fig. 5B, lane 8) or both (Fig. 5C, lanes 5 and 6) showed no detectable replication. Incorporating frameshift mutations either in UL102 (pZP19FR) or in UL105 (pZP20FR) also abrogated complementation (Fig. 5C, lanes 7 and 9, respectively). Thus, regions containing ORFs UL101-102 and UL105 were necessary and sufficient to replace pZP15.

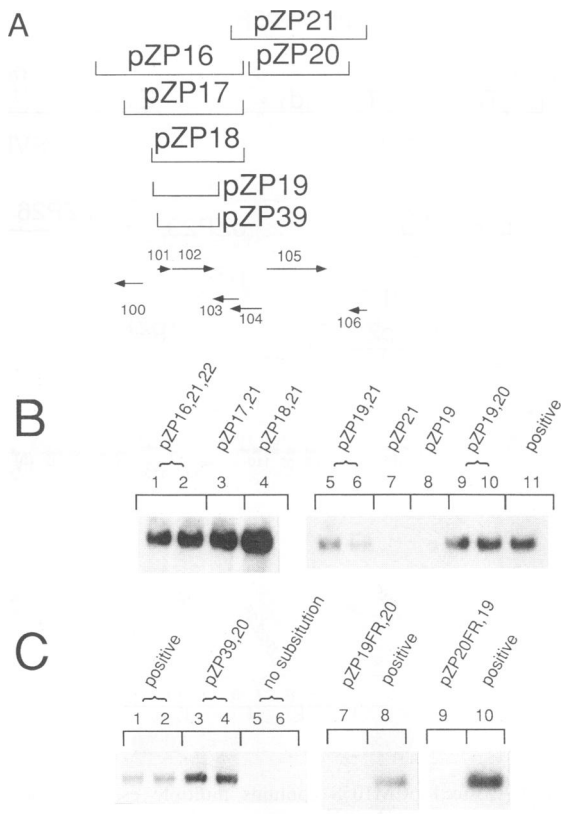

FIG. 5. Evidence that UL101-102 and UL105 are required. (A) Physical map of pZP15 subclones used in cotransfection experiments shown below and the corresponding ORFs. (B and C) Autoradiogram of a Southern transfer showing *Dpn*I-resistant *ori*Lyt-containing products from cotransfection mixtures containing pCM1017, pCM1029, pZP13, pZP3, pSVH, pZP22, and pSP50 plus the indicated plasmids substituted for pZP15.

To establish the required components in the right half of pCM1058, we made subclones of pZP22. Cotransfections substituting pZP23, which spans only ORFs UL112 to UL114, for pZP22 complemented replication (Fig. 6B, lanes 1 and 2). To determine which of these genes was required, we constructed two subclones of pZP23: pZP24, which lacks UL114, and pZP25, which contains only UL114. pZP24 was sufficient to replace pZP22 (Fig. 6B, lanes 5 and 6), whereas pZP25 alone did not (Fig. 6B, compare lanes 3 and 4 with lanes 7 and 8). Thus, ORFs UL112 and UL113 were the only pZP22 ORFs required. Also, pPSV (65), which expresses only the transcription unit spanning ORFs UL112 and UL113, replaced pZP24 (Fig. 6B, lanes 11 and 12). Finally, when subclones containing UL112-113 were omitted from transfection cocktails, no signal was detected (Fig. 6B, lanes 7, 8, 13, and 14). We concluded from the results summarized in this section that cosmid pCM1058 supplies four essential loci, containing ORFs UL101-102, UL105, UL112-113, and IE1 and IE2.

The above-described experiments identified eight required loci (see Fig. 7) in addition to the three that were described previously. In all, 11 distinct loci were needed to complement DNA replication in the cotransfection assay, as summarized in Table 1. To simplify the transfection mixtures for experiments defining the essential components of individual cosmids, we always included cosmid clones to provide some of the essential loci. Because of the possibility that other nonessential genes might contribute to DNA replication, we determined as a final test whether the defined set of 11 plasmid clones supplying only individual essential loci could support complementation. As shown in Fig. 6B, lanes 15 and 16, when only the defined set of 11 loci were cotransfected, *ori*Lyt replication was observed.

DISCUSSION

Transient complementation with cotransfected cloned fragments provides a genetic test of the role of previously identified candidate replication genes, as well as a means to identify other functions required for DNA synthesis (10, 29, 50). Moreover, because in our experiments essential replication functions are expressed via their native promoters, it also provides an approach to defining genetic requirements for

TABLE 1. Summary of essential HCMV loci and herpesvirus counterparts

Cosmid	HCMV	Herpesvirus counterpart		Predicted function
		HSV-1	Epstein-Barr virus	
pCM1017	UL44	UL42	BMRF1	*pol* accessory
	UL36-38	—[a]	—[b]	Regulatory
pCM1029	UL54	UL30	BALF5	DNA polymerase
	UL57	UL29	BALF2	Single-stranded-DNA-binding protein
	UL70	UL52	BSLF1	Primase
pCM1058	UL105	UL5	BBLF4	DNA helicase
	UL101-102	UL8[c]	BBLF2/3[c]	Primase-associated factor
	UL112-113	—[d]	—[d]	Unknown early protein
	IE1/IE2	—[a]	—[b]	Regulatory
pCM1052	IRS1	—[a]	—[b]	Regulatory
pCM1039	UL84	—[d]	—[d]	Unknown early protein

[a] HSV-1 transactivators are not required to complement DNA replication in transient assays.
[b] Three Epstein-Barr virus transactivators, Z, R, and M, are needed to complement *ori*Lyt replication in a transient assay; however, these genes are not obvious homologs of the essential HCMV transactivators.
[c] Probable homologs displaying minimal sequence similarity.
[d] No counterparts of these HCMV early temporal-class proteins have been found.

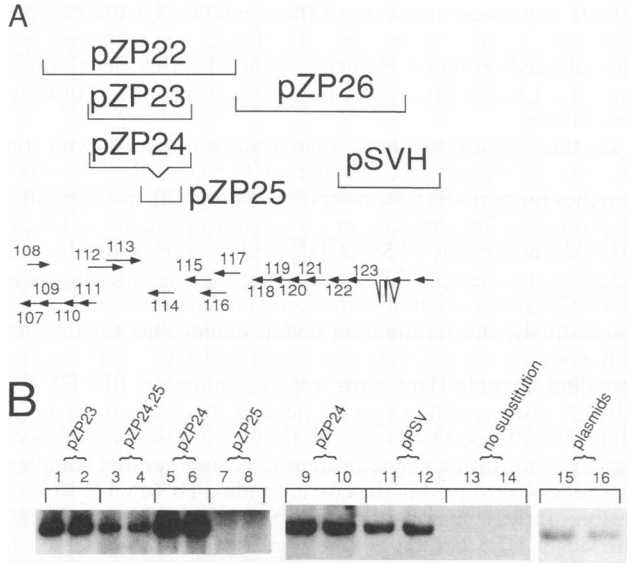

FIG. 6. Cosmid pCM1058 also contributes UL112-113. (A) Map of pZP38 subclones used in cotransfection experiments and the corresponding ORFs. (B) Transient-complementation assays carried out as described in Materials and Methods. Shown are autoradiograms of resulting Southern transfers, showing *Dpn*I-resistant *ori*Lyt-containing products. Cotransfection mixtures for lanes 1 to 12 contained pCM1017, pCM1029, pZP13, pZP3, pZP19, pZP20, pSVH, and pSP50 plus the indicated plasmids substituted for pZP22. Transfection mixtures for lanes 15 and 16 contained pZP3, pZP6, pZP7, pZP33, pZP28, pZP29, pZP13, pZP39, pZP20, pZP24, pSVH, and pSP50.

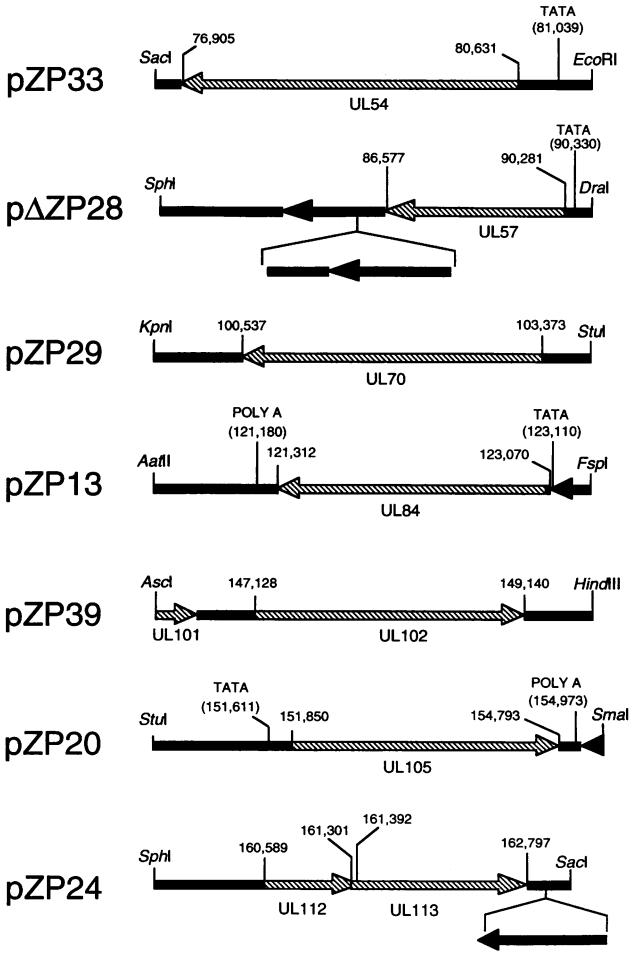

FIG. 7. HCMV fragments defining essential loci. The fragment present in each essential plasmid described in the text is drawn. The predicted essential ORFs are indicated, with hatched arrows showing the direction of transcription. Other partial ORFs also present in the fragments and deleted ORFs are shown as solid boxes. Where present, TATA and polyadenylation consensus sequences are also marked. The major immediate-early region, contributed in most of our experiments by pSVH (58), is not shown.

normal expression. We previously showed that UL44, UL36-38, and IRS1/TRS1 are required to complement HCMV *ori*Lyt-dependent DNA replication when the required genes are expressed from their native promoters (50). The results presented here identify the remaining essential loci. As diagrammed in Fig. 7 and detailed below, these additional loci span ORFs UL54, UL57, UL70, UL84, UL101-102, UL105, UL112-113, and the major immediate-early regions IE1 and IE2. Thus, altogether, 11 distinct loci are required for transient complementation of HCMV origin-dependent DNA replication.

Replication genes. Six of the essential loci span homologs or probable homologs of HSV-1 replication genes, encoding the DNA polymerase (UL54) and a polymerase-associated protein (UL44), the single-stranded-DNA-binding protein (UL57), and proteins that may form a helicase-primase complex (UL105, UL101-102, and UL70). Constructs with frameshift mutations within each of these ORFs individually failed to complement replication. Our results therefore provide genetic evidence consistent with a requirement for the proteins encoded by HCMV genes previously identified by nucleotide sequence similarity to HSV-1 replication genes. This finding is not surprising given the established sequence and biochemical similarities to HSV counterparts (4, 6, 12, 24). Candidate homologs of these proteins have been identified in all herpesviruses sequenced to date (1, 8, 12, 18, 48), and it is likely that these enzymes constitute a characteristic herpesvirus lytic-phase replicative apparatus. Supporting this is the finding that Epstein-Barr virus homologs of HSV-1 replication genes are also essential for complementation of lytic-phase DNA synthesis (29).

HCMV UL54 encodes a DNA polymerase that shows sequence similarity to a variety of alpha-like DNA polymerases (61) and is the target of currently available chemotherapeutic agents (14, 60). The coding assignment initially was based on sequence similarity to HSV-1 *pol* (37, 41). Earlier studies identified and characterized a DNA polymerase activity present in HCMV-infected cells (38, 39). Cloned and expressed UL54 demonstrates DNA polymerase activity in vitro that corresponds to the infected-cell enzyme (24, 25). Northern (RNA blot) analysis, nuclease protection, and primer extension assays suggested that the primary transcript expressing HCMV UL54 is a roughly 5-kb early species whose cap site is more than 380 nt upstream of the probable initiation codon (41) and that a polyadenylation signal lying within UL53 at nt 76330 is utilized. Our results are consistent with these observations. The smallest tested fragment supplying UL54 extends about 800 bp upstream of the predicted translation initiation codon (Fig. 7, pZP33) and just over 400 bp upstream of the experimentally determined cap site (41), demonstrating that a fibroblast-competent promoter sufficient to drive expression of UL54 is situated between nt 81011 and 81435. Deletions downstream of UL54 which eliminated the UL53 ORF also

removed the candidate polyadenylation signal, and these constructs failed to complement replication unless another polyadenylation sequence was substituted, as in pZP33. Thus, the previously noted signal is probably important for normal expression in vivo.

HCMV UL57 encodes a single-stranded-DNA-binding protein homologous to the HSV-1 major DNA-binding protein (3, 40). The transcript expressing UL57 is a 10- to 12-kb early species (40). The 5′ end of this transcript and its promoter are to the left of the *Dra*I site at nt 90842, as defined by the smallest complementing clone, although the in vivo cap site has not been determined. There is no downstream polyadenylation consensus sequence between UL57 and UL54. We found that in order to obtain complementation, extensive downstream sequence was required (pZP28). However, two lines of evidence indicate that UL57 is the only essential gene within this fragment. First, deletions in ORFs UL55 and UL56 did not eliminate complementation, in contrast to deletions or frameshift mutations within UL57. Second, we found that clones containing HCMV UL57 could be replaced by a clone expressing its CMV strain Colburn counterpart, which does not contain these downstream ORFs (2, 3).

UL70 encodes a homolog of HSV-1 UL52. UL52 contributes an essential primase activity to the HSV-1 three-subunit helicase-primase complex (16, 17, 22, 23). HCMV UL70 extends from nt 103373 to 100536 and is not flanked by consensus TATA or polyadenylation signals. In Northern blots, an approximately 4-kb transcript is detected (51), but the cap site has not been determined. The finding that pZP29 expresses UL70 also shows that the upstream boundary of the UL70 promoter must lie to the left of nt 103827, within about 450 bp of the predicted translation initiation codon. UL105, which extends from nt 151856 to 154793, encodes a homolog of HSV-1 UL5. Unlike UL70, UL105 is flanked by TATA and polyadenylation consensus sequences at nt 151611 and 154976, respectively. Within UL105 are characteristic helicase motifs similar to those noted in the corresponding helicases of HSV-1, varicella-zoster virus, and Epstein-Barr virus (46, 67). UL105 was supplied in our complementation assay by the subclone pZP20, which includes the candidate TATA and polyadenylation signals. This is the only ORF present in this clone. The HSV-1 counterparts of HCMV UL70 and UL105 associate with another HSV-1 gene product, UL8, to form a heterotrimeric helicase-primase complex (9, 17, 22, 23, 55). It remains to be determined whether HCMV UL70 and UL105 associate to form a similar complex.

The HCMV ORFs UL101 and UL102 were noted on the basis of flanking homologies to be positional counterparts of HSV-1 UL9 and UL8, respectively, although alignments with these counterparts were not obviously significant (12). In our assay, a construct spanning UL101 and UL102 was required for complementation (Fig. 5C, lanes 1 to 6). No obvious TATA or polyadenylation sequences are present in this fragment, and the transcription unit(s) is not well defined. UL102 must encode all or part of an essential function, because frameshift mutations in HCMV UL102 abrogated complementation (Fig. 5C, lane 7). cDNAs obtained by a polymerase chain reaction method (31) locate candidate 5′ ends of UL102 transcripts clustered around nt 146850, about 270 nt upstream of the predicted UL102 initiation codon (12). Only 7 nt 5′ with respect to the putative translational start of UL101 are present in the smallest complementing fragment tested (pZP39), and it seems unlikely that a transcript spanning UL101 is efficiently expressed. However, constructs in which the UL101 coding region is eliminated failed to complement replication, and two different frameshift mutations in the C-terminal portion of UL101, both well upstream of the candidate UL102 cap site, abrogated complementation (51). These constructions may have ablated promoter elements required for efficient expression of UL102 in fibroblasts, but we have not ruled out other possibilities.

In this regard we note that results obtained with the Genetics Computer Group program TESTCODE (21), which searches for periodic base composition bias (28), are suggestive of protein-coding capacity in the region upstream of UL102 (51). Moreover, this TESTCODE peak is approximately coincident with a 222-bp ORF, overlapping UL101, which begins at nt 146520 and is situated in the same reading frame as UL102. Interestingly, the termination codon ending this ORF is the only nonsense codon lying between it and UL102. The corresponding Epstein-Barr virus region, spanning BBLF2 and BBLF3, also was shown to be needed to complement DNA replication. These ORFs apparently are expressed via a spliced transcript to form a single protein (26), and parallel complexities may be observed in the corresponding HCMV UL101-102 region. It was suggested that Epstein-Barr virus BBLF2/3 encodes a UL8 homolog, and a small domain, conserved in HCMV UL102, was described (29).

Other required loci. A reasonable hypothesis is that the above-described six HCMV homologs of HSV-1 replication genes, shown in this report to be required for complementation, encode proteins that participate directly in DNA synthesis. Indeed, the only remaining unidentified or missing HCMV homolog of required HSV-1 replication genes is UL9. However, several other loci also were required in our assay. As in Epstein-Barr virus and distinct from HSV-1, three viral transactivators are needed for transient complementation of HCMV DNA replication. The finding that the major immediate-early locus is required for complementation is not surprising because expression of at least three of the above-described essential genes is upregulated by major immediate-early proteins (56–58). IRS1/TRS1 also activates expression of required proteins, in cooperation with major immediate-early proteins (57). Whether the required transactivators play other roles in regulating, initiating, or performing DNA synthesis remains to be determined.

An important emerging story concerns the roles of transactivators in regulating initiation of DNA replication. It is well established that origin usage in many eukaryotic and viral systems is augmented or controlled by elements that also regulate transcription (for reviews, see references 19 and 20), including HSV-1 and Epstein-Barr virus (33, 54, 63). Complementation of HSV-1 replication does not require any of the virus-coded transactivators (11), but HSV-1 ori_S activity is augmented by flanking promoter sequences (63). In contrast to HSV-1, complementation of Epstein-Barr virus *ori*Lyt-mediated replication requires three known viral transactivators (29, 52). One of these transactivators, Z, binds specifically to elements (ZREs) in the promoters of BHLF1 and BHRF1 that overlap *ori*Lyt (27, 44, 45). The BHLF1 promoter, including the ZREs, is an essential component of *ori*Lyt (52, 54).

The mechanisms by which transcriptional regulators influence origin activity are not established, but accumulating evidence suggests that, in some cases, activation is mediated by specific protein-protein interactions between transcription factors and replication proteins (36, 43). That there are both general and specific structural similarities between the lytic-phase origins of Epstein-Barr virus and HCMV (5, 47, 54) suggests that one or more of the HCMV transactivators may participate in activating HCMV *ori*Lyt. Now that the complete set of genes required for complementation has been established, it should be possible to distinguish whether individual

transactivators are required for initiation by placing the essential genes under the control of a heterologous constitutive promoter.

Finally, the functions of two other loci encoding early proteins required in the complementation assay, spanning ORFs UL84 and UL112-113, are not known; neither UL84 nor UL112-113 has been implicated previously in HCMV DNA replication or in regulating gene expression. UL84 codes for an early temporal class, nuclear, 65-kDa protein (35). Its 2.0-kb transcript is detected as early as 2.5 h postinfection, but not in cycloheximide-treated cells. Peak expression occurs later, between 72 and 96 h postinfection. The cap site is located immediately upstream of the translational start codon and just downstream from a TATA-like element at nt 123110. A polyadenylation signal is located downstream of UL84 at nt 121180. Both of these elements are present in pZP13, the smallest complementing clone tested. The complex transcription unit spanning ORFs UL112 and UL113 encodes at least four early temporal class, nuclear phosphoproteins of 34, 43, 50, and 84 kDa by differential splicing (64, 65). cDNA analysis has shown that 2.1- and 2.2-kb RNAs express the 50- and 43-kDa proteins, respectively, and that a 2.5-kb RNA expresses a 84-kDa protein; the 34-kDa protein is derived from an unspliced RNA. The promoter for these transcripts lies between −323 and −7 bp relative to the transcriptional start site and is activated by proteins originating from immediate-early regions 1 and 2 (56).

Homologs of UL84 and UL112-113 are not apparent in the genomes of the alpha- or gammaherpesviruses sequenced to date (1, 8, 18, 48), but moderate-stringency hybridizations with the UL84 and UL112-113 loci as probes detect specific fragments in the simian CMV-like strain Colburn (51). It is tempting to speculate that one or both of these loci replace the missing UL9 function, but it is equally plausible that these proteins supply additional transactivating functions or play some other unanticipated role. Finally, because we cannot rule out the possibility that the requirement for these proteins results from the transient-transfection assay used to identify them, it will be necessary to confirm the roles of these proteins by other genetic and biochemical approaches.

In summary, this study provides genetic evidence that, as expected, HCMV homologs of HSV-1 replication genes are essential for DNA replication. Like Epstein-Barr virus, HCMV evidently does not encode a homolog of HSV-1 UL9. Also as in Epstein-Barr virus, transient complementation of HCMV lytic-phase DNA synthesis requires three virus-coded transactivators. However, unique to HCMV is the apparent requirement for two additional early loci, UL84 and UL112-113. The results presented here lay the groundwork for future studies to define the roles of individual components in regulating, initiating, and performing DNA synthesis.

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