

Open Reading Frames UL44, IRS1/TRS1, and UL36-38 Are Required for Transient Complementation of Human Cytomegalovirus *oriLyt*-Dependent DNA Synthesis

GREGORY S. PARI,¹ MARILYN A. KACICA,² AND DAVID G. ANDERS^{1*}

Laboratory of Virology, The Wadsworth Center for Laboratories and Research, New York State Department of Health, and Department of Biomedical Sciences, SUNY School of Public Health, Albany, New York 12201-0509,¹ and Department of Pediatrics, Albany Medical College, Albany, New York 12201²

Received 18 November 1992/Accepted 19 January 1993

Previous results showed that plasmids containing human cytomegalovirus (HCMV) *oriLyt* are replicated after transfection into permissive cells if essential *trans*-acting factors are supplied by HCMV infection (D. G. Anders, M. A. Kacica, G. S. Pari, and S. M. Punturieri, *J. Virol.* 66:3373-3384, 1992). We have now used *oriLyt* as a reporter of HCMV DNA replication in a transient complementation assay in which cotransfected cosmid clones, instead of HCMV infection, provided essential *trans*-acting factors. Complemented replication was *oriLyt* dependent and phosphonoformic acid sensitive and produced tandem arrays typical of HCMV lytic-phase DNA synthesis. Thus, this assay provides a valid genetic test to find previously unidentified genes that are essential for DNA synthesis and to corroborate functional predictions made by nucleotide sequence comparisons and biochemical analyses. Five cosmids were necessary and sufficient to produce origin-dependent DNA synthesis; all but one of these required cosmids contain at least one candidate homolog of herpes simplex virus type 1 replication genes. We further used the assay to define essential regions in two of the required cosmids, pCM1017 and pCM1052. Results presented show that UL44, proposed on the basis of biochemical evidence to be the HCMV DNA polymerase accessory protein, was required for complementation. In addition, three genomic regions encoding regulatory proteins also were needed to produce origin-dependent DNA synthesis in this assay: (i) IRS1/TRS1, which cooperates with the major immediate-early proteins to activate UL44 expression; (ii) UL36-38; and (iii) the major immediate-early region comprising IE1 and IE2. Combined, these results unequivocally establish the utility of this approach for mapping HCMV replication genes. Thus, it will now be possible to define the set of HCMV genes necessary and sufficient for initiating and performing lytic-phase DNA synthesis as well as to identify those virus genes needed for their expression in human fibroblasts.

The set of human cytomegalovirus (HCMV) genes that carry out lytic-phase DNA replication has not been established, largely because of difficulties in performing genetic studies in this system. Therefore, much of our understanding of HCMV DNA replication has been gleaned by comparison with the prototype herpesvirus, herpes simplex virus type 1 (HSV-1). Challberg and colleagues defined seven HSV-1 genes as necessary and sufficient for origin-dependent DNA replication by using a transient cotransfection assay to test the ability of cloned fragments of HSV DNA to support the replication of an origin-containing plasmid (9-11, 63). These genes encode a DNA polymerase (UL30) and an associated polymerase accessory protein (UL42), a single-stranded-DNA-binding protein (UL29), a heterotrimeric helicase-primase complex (UL5, UL8, and UL52), and an origin-binding protein (UL9) (reviewed in references 10 and 11). Their results proved to be a nexus for extensive genetic and biochemical studies (e.g., references 8, 24, 42, 44, 50, 51, and 61; reviewed in reference 60). Candidate HCMV homologs of four of these HSV-1 replication genes, including a DNA polymerase (UL54), a single-stranded-DNA-binding protein (UL57), and two subunits of the helicase-primase complex (UL105 and UL70), were identified by sequence similarity (12). In addition, HCMV UL44 was identified as a candidate polymerase accessory protein on the basis of

biochemical studies of the expressed protein (18, 19). HCMV open reading frames (ORFs) positionally equivalent to HSV-1 UL8 and UL9 are not detectably similar in sequence (12). Protein products of ORFs UL57, UL54, and UL44 have been purified and characterized to various degrees (1, 3, 18, 19, 31, 34, 35), but except for the DNA polymerase, there has been little direct evidence that these candidate homologs are required for viral DNA synthesis *in vivo*.

It is important to better define all of the components of HCMV replication, because they confer the unique range of sensitivities exhibited for several antiviral agents. In addition, these components may provide useful targets for the rational design of new anti-HCMV chemotherapeutic agents. There may be significant differences between the components of HCMV DNA replication and those of other herpesviruses, despite the identification of candidate HCMV homologs of several of the HSV-1 replication genes and observed similarities in the DNA replication process. For example, one conspicuous gap is the failure to identify by sequence similarity an HCMV homolog of the HSV-1 origin-binding protein (12). This absence is particularly intriguing in the light of the distinctive structure of an HCMV origin of lytic-phase DNA replication, *oriLyt* (2, 4, 29, 43). In this regard, it was recently suggested that the gammaherpesvirus Epstein-Barr virus (EBV) lacks an equivalent of HSV-1 UL9 (20). Finally, some essential HCMV replication genes may

* Corresponding author.

be unique in that other herpesviruses do not encode homologs.

Our laboratory (2, 4) and others (29, 43) recently described HCMV *oriLyt*. HCMV *oriLyt* replicates autonomously when transfected into permissive cells if essential *trans*-acting factors are supplied by HCMV infection, which has facilitated its characterization (2, 4, 43). This finding suggested to us that the cotransfection strategy described by Challberg (9) could be used to locate essential HCMV replication genes. In this report, we describe a transient complementation assay in which *trans*-acting factors necessary for HCMV lytic-phase DNA replication are provided by a set of overlapping cosmids. We further describe the use of this assay to show that HCMV UL44, a proposed homolog of the HSV-1 polymerase accessory protein (18), is required for complementation of origin-dependent DNA replication. Moreover, IRS1/TRS1, a recently described transcription activator that acts cooperatively with HCMV immediate-early (IE) proteins to upregulate the UL44 promoter (55), also was indispensable, as were two other regions encoding previously described HCMV transcriptional activators, the major IE (MIE) regions 1 and 2, and ORFs UL36-38.

MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblast (HFF) cells were used for all experiments and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum. HCMV strain AD169 (American Type Culture Collection) was maintained as frozen stocks. Infection was done at an approximate multiplicity of infection of 10. Phosphonoformic acid (PFA; Sigma Chemical Co., St. Louis, Mo.) was used at a final concentration of 200 μ g/ml and added either at 24 h posttransfection or at the time of infection, as indicated.

Plasmid constructs. Construction of the replication reporter pSP50 was described previously (2); it contains HCMV *oriLyt* as a 5.6-kbp *PvuII*-to-*KpnI* subfragment of *HindIII*-D. HCMV cosmid clones (21) were kindly supplied by A. Colberg-Poley with the permission of B. Fleckenstein. Subclones of these cosmids all were constructed in pGEM7ZF(-) (Promega, Madison, Wis.) by using standard procedures (52). pSVH contains HCMV IE1 and IE2 (56) and was a gift of R. Stenberg. A clone containing strain AD169 TRS1 (pXEXX-6.1) was obtained from T. Jones (36), and plasmids containing strain Towne TRS1 (pON2334) and TRS1 with a frameshift mutation (pON2336) were supplied by E. Mocarski (55). Additional plasmid constructs were made as detailed below.

(i) **Subclones of cosmid pCM1052.** Plasmid pZP1 was made by excising from a low-melting-point (LMP) agarose gel the 20-kbp *HindIII* G fragment derived from pCM1052 and ligating it into pGEM7ZF(-). Plasmid pZP2 was made by excising the 12.6-kbp *EcoRI* F fragment from an LMP gel and ligating it into pGEM7ZF(-). Plasmid pZP3 was made by ligating the 3.6-kbp *SmaI* fragment extending from nucleotides (nt) 189500 to 193108 into pGEM7ZF(-).

(ii) **Subclones of pCM1017.** Plasmid pZP4 was made by ligating into pGEM7ZF(-) the 10.3-kb *HindIII* M fragment. pZP5 was made by excising the 7.0-kb *SacI* fragment extending from nt 52260 to 59219 from an LMP gel and ligating it into pGEM7ZF(-). pZP6 was made by excising the 8.0-kbp *BglII* fragment extending from nt 47366 to 56170 from an LMP gel and ligating it into pGEM7ZF(-). pZP7 was made by ligating the 2.6-kbp *KpnI* fragment extending from nt 54118 to 56701 into pGEM7ZF(-). p Δ ZP7 is pZP7 with a

582-bp *HincII* deletion extending from nt 54231 to 54813 that removes 429 nt of UL42 and 432 nt of UL43. pZP8 was made by ligating the 11-kbp *KpnI* fragment extending from nt 42045 to 53104 into pGEM7ZF(-).

Transfection and replication assay. HFF cells were plated onto 6-cm-diameter dishes at a cell density of 10^5 per plate 24 h before transfection. Approximately 4 h prior to transfection, the media were replaced with 3 ml of fresh DMEM. DNA was transfected by using the procedure described by Chen and Okayama (14). Briefly, 4 μ g of pSP50, 1 μ g each of cosmids pCM1017, pCM1029, pCM1039, pCM1035, pCM1052, pCM1058, and pCM1015, and 1 μ g of pSVH were added to a 5-ml sterile test tube. To this DNA mixture was added 0.5 ml of 0.25 M CaCl_2 followed by 0.5 ml of 2 \times BBS [50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 mM NaCl, 1.5 mM Na_2HPO_4 , (pH 6.95)]. The solution was vortexed briefly and allowed to stand at room temperature for 20 min; 0.5 ml of this cocktail was added dropwise to each plate, and the dishes were incubated at 37°C in a 3% CO_2 incubator for approximately 20 h. Dishes then were washed twice with DMEM, 3 ml of fresh DMEM containing 10% fetal calf serum was added, and cells were further incubated at 37°C in a 5% CO_2 incubator for up to 120 h. All transfections were done in duplicate. A typical positive control for these experiments, not usually depicted in the results presented, was a parallel transfection with the complete set of cosmids plus pSP50, followed by infection 24 h after transfection.

Total cell DNA was collected by removing the media, washing cells once with phosphate-buffered saline, and lysing cells directly on the dish with 400 μ l of cell lysis buffer (2% sodium dodecyl sulfate, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA). Cell lysates were transferred to a 1.5-ml microcentrifuge tube, proteinase K was added to a final concentration of 200 μ g/ml, and the lysates were incubated at 50°C for 3 h. Sodium acetate (pH 5.4) was added to a final concentration of 0.3 M; the cell lysates were then extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1) and ethanol precipitated. DNA was resuspended in 100 μ l of Tris-EDTA (pH 8.0), and one-quarter of the total was treated with 25 U of *EcoRI* and 10 U of *DpnI* in a volume of 50 μ l at 37°C for 4 to 16 h. Cleaved DNA was electrophoresed through a 0.8% agarose gel, transferred to a Zeta-probe (Bio-Rad, Richmond, Calif.) nylon membrane, and probed with random-primer ^{32}P -labeled pGEM. Hybridizations were performed according to manufacturers' instructions. Southern blots were then exposed to X-Omat AR X-ray film (Kodak) at -80°C for 24 h.

RESULTS

HCMV cosmid clones can direct lytic-phase DNA replication when cotransfected with a plasmid which expresses IE1 and IE2. In developing a transient complementation assay for HCMV DNA replication, our strategy was to introduce large overlapping cloned segments of the HCMV genome into permissive HFF cells together with a plasmid containing HCMV *oriLyt*. Available cosmid libraries (21) provided convenient sets of overlapping genomic fragments, and the *oriLyt*-containing plasmid SP50 (4) served as an assayable reporter of *oriLyt*-mediated DNA synthesis; replication of pSP50 was detected by treatment of transfected-cell DNA with *DpnI*, which cleaves only unreplicated *dam*-methylated input DNA. Initial experiments tested various combinations of available cosmids, plus or minus added pSVH, which

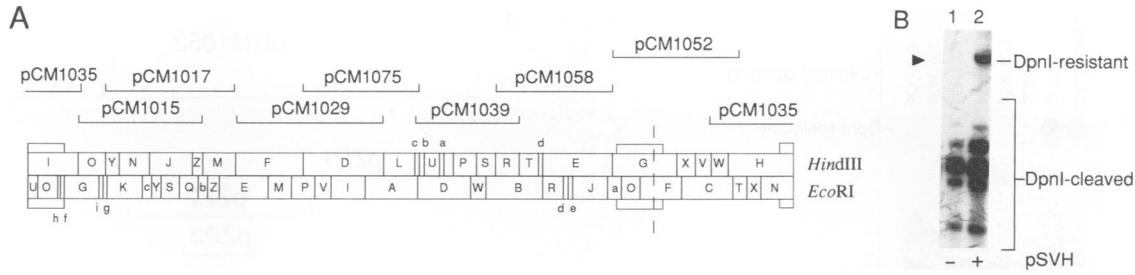


FIG. 1. Ability of cotransfected cosmids to complement replication of cloned HCMV *oriLyt*. (A) Physical maps of the HCMV genome and locations of the cosmids used for the described experiments. (B) Evidence that cosmid complementation requires pSVH. Cotransfection was done as described in Materials and Methods. Cotransfection mixtures contained 4 μ g of pSP50 and 1 μ g each of cosmids pCM1015, pCM1017, pCM1029, pCM1039, pCM1058, pCM1052, and pCM1035 (lane 1) or the same set of recombinants plus 1 μ g of pSVH (lane 2). Shown is an autoradiogram of the resulting Southern transfer. The arrowhead indicates the position of the pGEM7Zf(-)-containing *EcoRI* fragment of the reporter plasmid pSP50.

contains the MIE regions 1 and 2 (56), and compared several transfection methods, including standard CaPO₄, DEAE-dextran and lipofection protocols. Although all of the transfection protocols were efficient enough for standard transfection experiments in which only *oriLyt* was transfected and necessary *trans*-acting factors were supplied by infection, we found that only the BES-buffered, modified CaPO₄ procedure described by Chen and Okayama (14) yielded complementation (47). Positive results, measured by conversion of *oriLyt* to *DpnI* resistance, first were obtained when the seven cosmids pCM1015, pCM1017, pCM1029, pCM1035, pCM1039, pCM1058, and pCM1052 were cotransfected with a roughly 20-fold molar excess of pSVH (Fig. 1B, lane 2). Thus, these seven partially overlapping cosmids expressed all of the proteins needed to direct detectable *oriLyt* replication. Together they comprise almost the entire HCMV genome (Fig. 1A), including all of the described homologs of HSV-1 replication genes. Without added pSVH, the same set of cosmids reproducibly failed to complement DNA replication (e.g., Fig. 1B, lane 1), even though pCM1058 contains a functioning MIE region (47). In this and other experiments, only *oriLyt*-containing plasmids were replicated. While it is difficult to interpret these results quantitatively, we note that in typical experiments, complemented replication produced a *DpnI*-resistant signal roughly 50- to 100-fold lower than that found for the parallel infected control (e.g., compare lanes 0 and 1 in Fig. 4C).

Two additional control experiments were done to test whether the detected *DpnI*-resistant products resulted from HCMV lytic-phase DNA synthesis. PFA treatment, which selectively inhibits the virus-encoded DNA polymerase, was used to determine whether the *oriLyt* replication resulting from cosmid cotransfection required HCMV DNA polymerase activity. HFF cells were cotransfected with the indicated set of clones and then incubated with or without PFA for up to 144 h (Fig. 2A). Replication of *oriLyt* was detected from 72 h after transfection in untreated cells (lanes 4, 6, 8, and 10). However, in PFA-treated cells, no *DpnI*-resistant product was detected at any time point (lanes 1, 3, 5, 7, and 9). This finding is consistent with results from earlier experiments showing the PFA sensitivity of *oriLyt*-mediated DNA replication (4). We also examined the structures of replication products resulting from cosmid cotransfection. Replication products of *oriLyt*-containing plasmids in the transient transfection-plus-infection assay are tandem arrays (4). Thus, partial digestion with an enzyme that cleaves the vector at a unique site produces a ladder of

fragments representing 1N, 2N, 3N, etc., copies of the reporter plasmid. Similar patterns were seen when *oriLyt* replication products from HCMV-infected (Fig. 2C) and cosmid-cotransfected (Fig. 2B) HFF cells were compared by partial digestion. Together, these results argue that *oriLyt* replication directed by cotransfected cosmids is equivalent to that of the transient transfection-plus-infection assay and accurately reproduces events of HCMV lytic-phase DNA replication.

Five cosmids are needed for complementation of HCMV origin-dependent DNA replication. Once it was established that the cotransfected cosmids directed authentic lytic-phase replication of *oriLyt*, we next identified which of the cosmids contained genes essential for transient complementation by eliminating one cosmid at a time from transfection mixtures. When cosmids pCM1015 and pCM1035 were omitted from cotransfection cocktails (Fig. 3, lanes 2 and 5, respectively), *oriLyt* replication was unaffected. *HindIII* fragments I, H,

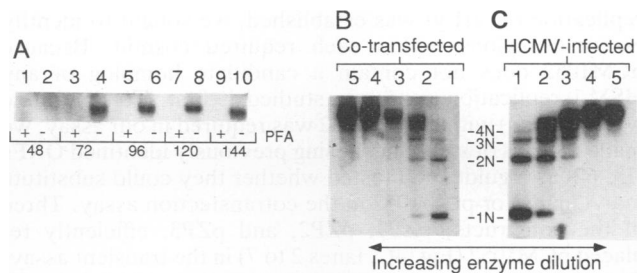


FIG. 2. Evidence that replication of *oriLyt* following cotransfection is authentic HCMV lytic-phase DNA synthesis. (A) Complementation is PFA sensitive. HFF cells were cotransfected with a mixture containing cosmids pCM1017, pCM1029, pCM1039, pCM1058, and pCM1052 plus plasmids pSVH and pSP50 and then incubated for the indicated time with (lanes 1, 3, 5, 7, and 9) or without (lanes 2, 4, 6, 8, and 10) 200 μ g of PFA per ml of medium. DNA was extracted and digested as described in Materials and Methods. Only that portion of the resulting Southern transfer containing the *DpnI*-resistant products is shown. (B and C) Complemented DNA synthesis produces tandem arrays of pSP50. Parallel sets of dishes were cotransfected with a replication-competent set of cosmids plus pSP50. One set of dishes was infected (C), and the other set was left uninfected (B); the dishes were incubated for 96 h, and total DNA was prepared as described in Materials and Methods. *DpnI*-resistant DNA was cleaved partially with *HindIII* by the serial dilution method.

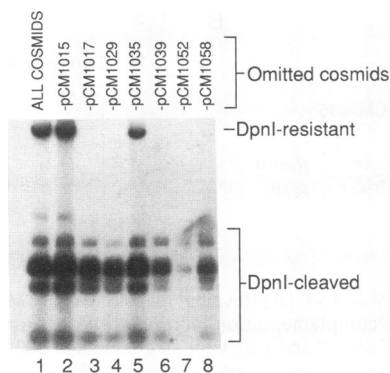


FIG. 3. Evidence that five cosmids are needed for complementation of HCMV origin-dependent DNA replication. Transient complementation assays were performed as described in Materials and Methods. All transfections contained pSP50 and pSVH plus either the complete set of seven cosmid clones (pCM1015, pCM1017, pCM1029, pCM1035, pCM1039, pCM1052, and pCM1058; lane 1) or the complete set of cosmid clones minus the indicated cosmid (lanes 2 to 8). Shown is the resulting Southern transfer. The position of *DpnI*-resistant replication products is indicated.

and O are unique to these cosmids (Fig. 1A); therefore these fragments do not express genes essential for origin-dependent HCMV DNA replication. However, no replication products were detected when any of the four cosmids containing previously identified candidate replication genes, pCM1017, pCM1029, pCM1039, and pCM1058, were omitted from the transfection cocktails (Fig. 3, lanes 3, 4, 6, and 8). In addition, cosmid pCM1052, which does not contain an identified homolog of an HSV-1 replication gene, also was required (Fig. 3, lane 7). Thus, five cosmids (pCM1017, pCM1029, pCM1039, pCM1052, and pCM1058) plus pSVH were needed to complement DNA replication in our transient assay.

IRS1/TRS1 is essential for transient complementation. Once the minimum set of cosmid clones sufficient to direct the replication of *oriLyt* was established, we sought to identify essential regions within each required cosmid. Because pCM1052 does not contain a candidate homolog of any HSV-1 replication gene, we studied it first. To determine which gene(s) within pCM1052 was required in our assay, we made a series of subclones, using previously identified ORFs (13, 62) as a guide, and tested whether they could substitute individually for pCM1052 in the cotransfection assay. Three of the constructs, pZP1, pZP2, and pZP3, efficiently replaced pCM1052 (Fig. 4C, lanes 2 to 7) in the transient assay. Transfections omitting pCM1052 without substitution produced no detected signal (Fig. 4C, lane 8), as shown above (Fig. 3, lane 7). None of the other tested fragments derived from pCM1052, including *HindIII*-W and -V, could substitute for pCM1052 (47). pZP3 contains a 3.6-kb *SmaI* fragment that spans only the ORFs IRS1 and US1 (Fig. 4A and B). Because US1 has been shown to be dispensable for virus growth in culture (36), we suspected that IRS1 was the essential ORF. TRS1, the terminal repeat counterpart of IRS1, shares extensive sequence identity with IRS1 (12), and both can cooperate with IE1 and IE2 to transactivate delayed-early gene expression (55). In light of the finding that the IRS1 region of pCM1052 was sufficient for complementation, it was surprising to us that cosmid pCM1035, which contains TRS1, failed to substitute for pCM1052 (Fig. 3, lane 7). However, we subsequently found that a plasmid con-

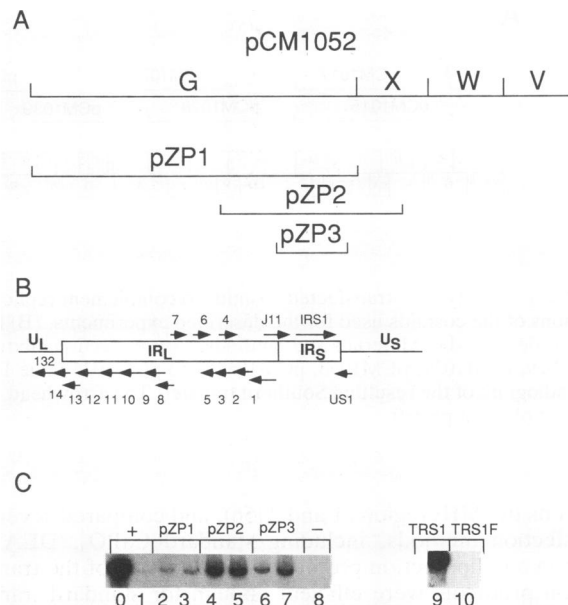


FIG. 4. Evidence that IRS1/TRS1 is essential for origin-dependent DNA replication. (A) Physical map depicting *HindIII* fragments within pCM1052 and fragments that were subcloned as described in Materials and Methods and then tested in cotransfection experiments. pZP1 extends from nt 176883 to 195837. pZP2 extends from nt 185495 to 195231. pZP3 extends from nt 189500 to 193108. (B) ORFs predicted by Chee et al. (12) situated within the IR region between the U_L and the U_S of HCMV. (C) Cotransfection experiments showing that IRS1/TRS1 is essential for complementation of DNA synthesis. Cotransfections contained pSP50, pSVH, and cosmids pCM1017, pCM1029, pCM1039, pCM1058, and pCM1052 (lanes 0 and 1), the same cocktail with the indicated substitution for pCM1052 (lanes 2 to 7, 9, and 10), or the same transfection mixture minus pCM1052 without substitution (lane 8). The dish transfected for the sample run in lane 0 was infected as described in Materials and Methods 24 h after transfection. In lanes 9 and lane 10, pON2334 (TRS1) and pON2336 (TRS1F), respectively, were substituted for pCM1052 (55) as explained in Results.

struct spanning HCMV strain AD169 TRS1 (pXEXX-6.1; provided by T. Jones) could substitute for cosmid pCM1052 following cotransfection (47); thus, the TRS1 copy present in pCM1035 is probably defective. Further supporting this contention was the finding that a plasmid containing only TRS1 (from HCMV strain Towne; pON2334; kindly provided by E. Mocarski) efficiently substituted for pCM1052 (Fig. 4C, lane 9) upon cotransfection, whereas a frame-shifted mutant of TRS1 (pON2336) lacked the essential activity (Fig. 4C, lane 10). We concluded that IRS1 is the only ORF within cosmid pCM1052 needed for transient complementation and that TRS1 can substitute for IRS1. Thus, except for IRS1/TRS1, the genomic segments containing U_S , IR_L, and IR_S were dispensable for lytic-phase DNA synthesis. Replication signals in these substituted transfections were higher than with pCM1052 (Fig. 4C; compare lane 1 with lanes 3 to 7), presumably because of the higher effective concentration of an essential gene that resulted from replacing pCM1052 with an equal mass of smaller plasmids. A related observation is that extramolar IRS1 relieved the observed requirement for pSVH, although the MIE region, supplied by pCM1058, was still essential (47).

Cosmid pCM1017 contains two regions essential for *oriLyt*-dependent DNA replication. IRS1/TRS1 recently was shown

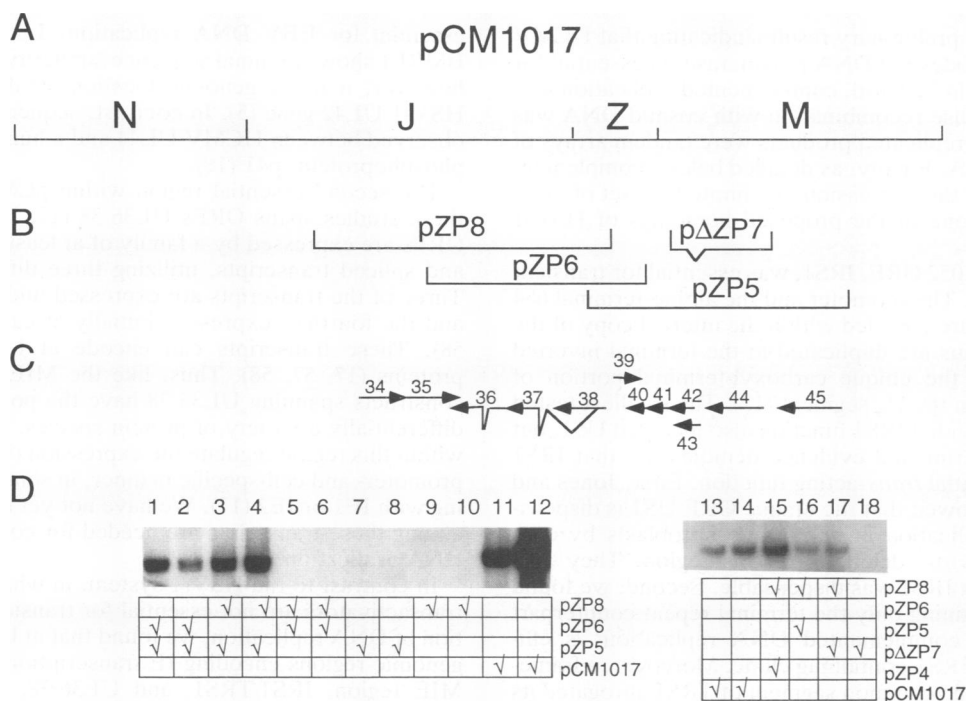


FIG. 5. Presence in cosmid pCM1017 of two regions that are required for origin-dependent DNA replication. (A) Physical map depicting *Hind*III fragments within cosmid pCM1017. (B) Fragments that were subcloned from cosmid pCM1017 and used in the described cotransfection experiments. Subclone pZP8 contains a fragment that extends from nt 42045 to 53104. pZP6 contains a fragment that extends from nt 47366 to 56170. pZP5 contains a fragment that extends from nt 52260 to 59219. pΔZP7 contains a fragment that extends from nt 54118 to 56701 with a *Hinc*II deletion from nt 54231 to 54813. (C) Predicted ORFs (12) within the essential region of pCM1017. (D) Cotransfection experiments defining essential regions within cosmid pCM1017. All transfections contained pSP50, pSVH, pZP3, pCM1029, pCM1039, and pCM1058, plus pCM1017 (lanes 11 to 14) or the substitution for pCM1017 indicated below each lane with a checkmark (lanes 1 to 10 and 15 to 18).

to *trans* activate HCMV UL44 expression in cooperation with the MIE region (55). Elsewhere, UL44 was proposed to encode a polymerase accessory protein on the basis of biochemical studies of the expressed protein (18), suggesting that it is a component of the DNA synthesis apparatus. Those findings predicted that UL44 should be essential for transient complementation of DNA synthesis. Therefore, to determine whether UL44 was required, we tested a subclone that left intact only the predicted transcription unit for UL44 (pΔZP7) and found that it failed to replace pCM1017 in the replication assay (Fig. 5, lane 18). Therefore, additional pCM1017 subclones were made and tested both individually and together with UL44-containing constructs (Fig. 5) (47). None of the individual constructs that were tested could replace pCM1017 (e.g., Fig. 5D, lanes 7 to 10). However, the combination of pΔZP7 (or pZP5) with either pZP6 or pZP8 (Fig. 5D, lanes 1 to 4, 16, and 17) substituted for pCM1017. The 582-nt *Hinc*II deletion in pΔZP7 removed all but the amino-terminal 41 nt of the 420-nt UL42 ORF as well as the carboxyl-terminal 209 nt of the 561-nt UL43 ORF, leaving only UL44 intact. Furthermore, a frameshifted mutant of UL44 in which the *Bgl*II site (nt 56170) was filled in with Klenow enzyme failed to complement replication (47), providing additional evidence that UL44 is the essential ORF in pΔZP7. The finding that either pZP6 or pZP8 could cooperate with pΔZP7 to replace pCM1017 defined a 5.7-kbp region extending from the *Bgl*II site at nt 47366 to the *Kpn*I site at nt 53104 and spanning only ORFs UL36-38. Moreover, a construct containing this 5.7-kbp fragment also substituted

for pCM1017 (47). Therefore, from this series of experiments, we concluded that both UL44 and the region spanning ORFs UL36-38 were needed to replace pCM1017 in the replication assay (Fig. 5D, lane 17).

DISCUSSION

We have developed a transient complementation assay of HCMV lytic-phase DNA replication that is based on the strategy devised by Challberg (9) and uses available cosmid libraries. Five cosmids, pCM1017, pCM1029, pCM1039, pCM1052, and pCM1058, plus pSVH, which comprises the MIE region, were required for complementation of DNA replication in permissive HFF cells. In this report, we demonstrate the validity of this assay and show results locating the genes essential for complementation in two of the required cosmids, pCM1017 and pCM1052.

Several lines of experimental evidence indicate that DNA synthesis resulting from transient complementation accurately reproduces events of HCMV lytic-phase DNA replication and thus is a valid approach to identifying HCMV replication genes. First, complemented replication was *ori*-Lyt dependent. For example, neither pSVH nor any of the cosmids not containing *ori*Lyt were replicated, although replication of pCM1029, which includes *ori*Lyt, was readily seen. Second, replication was PFA sensitive, as is viral DNA replication (4), arguing that the HCMV-contributed DNA polymerase is essential for complementation of lytic-phase DNA synthesis, as would be predicted. Consistent

with this view are preliminary results indicating that HCMV UL54, which encodes the DNA polymerase, is essential for complementation (47). Third, complemented replication was autonomous because recombination with cosmid DNA was not detected, and replicated products were tandem arrays of input plasmid DNA. Finally, as detailed below, complementation depends on the expression of a limited subset of virus genes, including one of the proposed homologs of HSV-1 replication genes.

Only one pCM1052 ORF, IRS1, was essential for transient complementation. The promoter and the amino-terminal 684 residues of IRS1 are encoded within the internal copy of the IR_S region and thus are duplicated in the terminal inverted repeat as TRS1; the unique carboxyl-terminal portion of IRS1 is encoded in the U_S segment (12). The smallest tested fragment that provided IRS1 function also included US1, but two lines of experimental evidence demonstrate that IRS1 encodes the essential *trans*-acting function. First, Jones and Muzithras (36) showed that the unique ORF US1 is dispensable for viral replication in permissive fibroblasts by constructing viable virus deletions of this region. They also demonstrated that IRS1 was dispensable. Second, we found that a clone containing only the terminal repeat counterpart of IRS1, TRS1, complemented DNA replication as efficiently as did the IRS1-containing clone. Moreover, a frameshift mutation in the common segment of TRS1 abrogated its ability to replace IRS1 (Fig. 4C, lanes 9 and 10). Thus, either IRS1 or TRS1 was essential to complement DNA replication, but the rest of the U_S and inverted repeat regions were dispensable. Our results are therefore consistent with the findings of Jones and Muzithras (36) and further suggest that it may not be possible to delete simultaneously both IRS1 and TRS1 because at least one copy may be essential for growth in fibroblasts. IRS1 and TRS1 have amino-terminal sequence similarity to several other predicted HCMV ORFs, which are grouped as the US22 family (12, 62), and we note that none of the genomic clones containing other members of this family that were tested, including those spanning UL36-38, could substitute for IRS1/TRS1 in our assay.

Two distinct regions within pCM1017 were needed to complement HCMV DNA replication. The two defined segments span ORFs UL44 and UL36-38, respectively. No other ORFs remained intact in the fragment used to provide UL44 (pΔZP7), suggesting that UL44 encodes the required protein. Moreover, a reading frame mutation near the amino terminus of UL44 abolished the ability of this fragment to complement replication, supporting this contention (47). UL44 encodes an abundant, phosphorylated DNA-binding protein of about 53 kDa (23, 45) that is expressed beginning in the early phase of infection and accumulates through the late phase (23). Ertl and Powell expressed both HCMV DNA polymerase and UL44 in insect cells by using recombinant baculoviruses and showed that the protein product of UL44 complexes with coexpressed polymerase (18). Moreover, UL44 selectively stimulated HCMV DNA polymerase activity in a template-dependent manner. On the basis of these results, they suggested that UL44 functions as a polymerase accessory protein, equivalent to HSV-1 UL42 (25, 33), although sequence similarity to HSV-1 UL42 is not readily apparent. Thus, our results, considered together with available biochemical evidence, are consistent with a model in which HCMV UL44 participates directly in DNA synthesis via physical interaction with other components of the replication machinery. Using a similar transient assay, Fixman et al. (20) recently demonstrated that EBV BMRF1, which associates with the EBV-coded polymerase (37, 38), is

essential for EBV DNA replication. Like HCMV UL44, BMRF1 shows minimal sequence similarity to HSV-1 UL42; however, it has a genomic location similar to that of the HSV-1 UL42 gene (5). In contrast, sequence similarity was observed between HCMV UL44 and a human herpesvirus 6 phosphoprotein, p41 (18).

The second essential region within pCM1017 defined by these studies spans ORFs UL36-38 (12, 17, 57, 58). These ORFs are expressed by a family of at least four overlapping and spliced transcripts, utilizing three different start sites. Three of the transcripts are expressed under IE conditions, and the fourth is expressed initially at early times (17, 57, 58). These transcripts can encode at least four distinct proteins (17, 57, 58). Thus, like the MIE region, genomic constructs spanning UL36-38 have the potential to express differentially a variety of protein species. Proteins encoded within this region regulate the expression of target genes in a promoter- and cell-specific manner, in some cases cooperating with IE1 or IE2 (17). We have not yet determined which among these transcripts are needed for complementation of DNA replication.

In contrast to the HSV-1 system, in which virus-encoded transactivators are not essential for transient complementation of DNA replication, we found that at least three distinct genomic regions encoding IE transcriptional regulators, the MIE region, IRS1/TRS1, and UL36-38, were needed for complementation of *ori*_{Lyt}-mediated DNA synthesis. The essential roles played by each of these three transactivators in transient complementation of DNA synthesis have not been established, but there are several nonexclusive possibilities. First, it is likely that these transactivators are needed to promote the expression of early genes required for HCMV DNA replication, because it has been shown that IRS1/TRS1 and the MIE regions encoding IE1 and IE2 can cooperate to activate expression of essential early genes in transient transfection assays (55). The MIE proteins have been studied extensively with respect to their role in regulating HCMV and heterologous promoters (6, 15, 22, 27, 32, 41, 49, 55, 56, 59). They upregulate expression from several delayed-early promoters, including those of the DNA polymerase and UL44 (54, 55, 56). Like the MIE region, TRS1 is expressed at IE times after infection and in the presence of cycloheximide (55). We found that IRS1/TRS1 was essential for transient complementation of DNA synthesis, whereas Stasiak and Mocarski (55) observed only a severalfold superinduction of UL44 by TRS1 plus the MIE region over the MIE region-activated UL44 promoter. However, we note that this effect would be multiplied if IRS1/TRS1 similarly induces expression of other genes essential for DNA synthesis, which could readily account for its key role in complementation. UL36-38 gene products also activate transcription of selected cellular genes as well as HCMV early genes in a cell-type-specific manner in cooperation with IE1 and IE2 (17). However, replication genes have not yet been shown to be targets for transcriptional activation by UL36-38. A second possibility is that these IE regions induce cellular factors that influence HCMV viral DNA replication. The corresponding MIE proteins of mouse cytomegalovirus and HCMV have been implicated in the induction of cellular DNA replication and transcription (27, 48, 53) and thus could play another crucial role by increasing the availability of needed host factors such as topoisomerase (7). Finally, it is also possible that one or more of the transactivators directly affects or regulates initiation of replication, as has recently been suggested for simian virus 40 and for the papillomaviruses (16, 26, 46). In this regard, *ori*_{Lyt}-dependent EBV

replication also requires three IE proteins, Zta, Rta, and Mta, although the requirement for Mta is not absolute (20). Zta binds directly to elements in two distinct domains of the DS_L promoter and enhancer regions and activates divergent transcription (30, 39, 40). This region lies within EBV *oriLyt* (28), and Zta may play a role in promoting EBV DNA replication.

Experiments are under way to identify specific genes within the remaining cosmids that contribute genes essential for DNA replication. Results obtained thus far are consistent with a requirement for candidate HCMV homologs of HSV-1 replication genes (47). Once all of the genes needed for complementation are identified, it will be possible to address experimentally the role of viral transactivators.

ACKNOWLEDGMENTS

We thank Rich Stenberg, Tom Jones, Ed Mocarski, and Annie Colberg-Poley for generous gifts of recombinant plasmids; J. Nelson for providing antibodies; and Mary Beth Coy for dedicated technical assistance.

This work was aided by Public Health Service NIH grants AI31249 and AI33416. G.S.P. is a Wadsworth postdoctoral fellow.

REFERENCES

- Anders, D. G., A. Irmiere, and W. Gibson. 1986. Identification and characterization of a major early cytomegalovirus DNA-binding protein. *J. Virol.* **58**:253-262.
- Anders, D. G., M. A. Kacica, G. S. Pari, and S. M. Punturieri. 1992. Boundaries and structure of human cytomegalovirus *oriLyt*, a complex origin for lytic-phase DNA replication. *J. Virol.* **66**:3373-3384.
- Anders, D. G., J. R. Kidd, and W. Gibson. 1987. Immunological characterization of an early cytomegalovirus single-strand DNA-binding protein with similarities to the HSV major DNA-binding protein. *Virology* **161**:579-588.
- Anders, D. G., and S. M. Punturieri. 1991. Multicomponent origin of cytomegalovirus lytic-phase DNA replication. *J. Virol.* **65**:931-937.
- Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, and G. Hatfull. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature (London)* **310**:207-211.
- Baracchini, E., E. Glezer, K. Fish, R. M. Stenberg, J. A. Nelson, and J. A. Ghazal. 1992. An isoform variant of the cytomegalovirus immediate-early auto repressor functions as a transcriptional activator. *Virology* **188**:518-530.
- Benson, J. D., and E. S. Huang. 1988. Two specific topoisomerase inhibitors prevent replication of human cytomegalovirus DNA: an implied role in replication of the viral genome. *J. Virol.* **62**:4797-4800.
- Carmichael, E. P., M. J. Kosovsky, and S. K. Weller. 1988. Isolation and characterization of herpes simplex mutants defective in viral DNA synthesis. *J. Virol.* **62**:91-99.
- Challberg, M. D. 1986. A method for identifying the viral genes required for herpes DNA replication. *Proc. Natl. Acad. Sci. USA* **83**:9094-9098.
- Challberg, M. D. 1991. Herpes simplex virus DNA replication. *Semin. Virol.* **2**:247-256.
- Challberg, M. D., and T. J. Kelly. 1989. Animal virus DNA replication. *Annu. Rev. Biochem.* **58**:671-717.
- Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, and T. Horsnell. 1990. Analysis of the coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* **154**:125-169.
- Chee, M. S., S. C. Satchwell, E. Preddie, K. M. Weston, and B. G. Barrell. 1990. Human cytomegalovirus encodes three G protein-coupled receptor homologues. *Nature (London)* **344**:774-777.
- Chen, C., and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745-2752.
- Cherrington, J. M., E. L. Khoury, and E. S. Mocarski. 1991. Human cytomegalovirus *ie2* negatively regulates α gene expression via a short target sequence near the transcription start site. *J. Virol.* **65**:887-896.
- Chiang, C. M., G. Dong, T. R. Broker, and L. T. Chow. 1992. Control of human papillomavirus type 11 origin of replication by the E2 family of transcription regulatory proteins. *J. Virol.* **66**:5224-5231.
- Colberg-Poley, A. M., L. D. Santomena, P. P. Harlow, P. A. Benfield, and D. J. Tenney. 1992. Human cytomegalovirus US3 and UL36-38 immediate-early proteins regulate gene expression. *J. Virol.* **66**:95-105.
- Ertl, P. F., and K. L. Powell. 1992. Physical and functional interaction of human cytomegalovirus DNA polymerase and its accessory protein (ICP36) expressed in insect cells. *J. Virol.* **66**:4126-4133.
- Ertl, P. F., M. F. Thomas, and K. L. Powell. 1991. High level expression of DNA polymerases from herpesviruses. *J. Gen. Virol.* **72**:1729-1734.
- Fixman, E. D., G. S. Hayward, and S. D. Hayward. 1992. *trans*-acting requirements for replication of Epstein-Barr virus *oriLyt*. *J. Virol.* **66**:5030-5039.
- Fleckenstein, B., I. Muller, and J. Collins. 1982. Cloning of the complete human cytomegalovirus genome in cosmids. *Gene* **18**:39-46.
- Ghazal, P., J. Young, E. Giuliotti, C. DeMattei, J. Garcia, R. Gaynor, and R. M. Stenberg. 1991. A discrete *cis* element in the human immunodeficiency virus long terminal repeat mediates synergistic *trans* activation by cytomegalovirus immediate-early proteins. *J. Virol.* **65**:6735-6742.
- Gibson, W. 1984. Synthesis, structure and function of cytomegalovirus major nonvirion nuclear protein, p. 423-440. *In* F. Rapp (ed.), *Herpesvirus*. Alan R. Liss, New York.
- Goldstein, D. J., and S. K. Weller. 1988. An ICP6::*lacZ* insertional mutagen is used to demonstrate that the UL52 gene of herpes simplex virus type 1 is required for virus growth and DNA synthesis. *J. Virol.* **62**:2970-2977.
- Gottlieb, J., A. I. Marcy, D. M. Coen, and M. D. Challberg. 1990. The herpes simplex virus type 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. *J. Virol.* **64**:5976-5987.
- Guo, Z.-S., and M. L. DePamphilis. 1992. Specific transcription factors stimulate simian virus 40 and polyomavirus origins of DNA replication. *Mol. Cell. Biol.* **12**:2514-2524.
- Hagemeyer, C., S. M. Walker, P. J. G. Sissons, and J. H. Sinclair. 1992. The 72K IE1 and 80K IE2 proteins of human cytomegalovirus independently trans-activate the *c-fos*, *c-myc* and *hsp70* promoters via basal promoter elements. *J. Gen. Virol.* **73**:2385-2393.
- 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.
- 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.
- Hardwick, J. M., P. M. Lieberman, and S. D. Hayward. 1988. A new Epstein-Barr virus transactivator, R, induces expression of a cytoplasmic early antigen. *J. Virol.* **62**:2274-2284.
- Heilbronn, R., G. Jahn, A. Burkle, U.-K. Freese, B. Fleckenstein, and H. zur Hausen. 1987. Genomic localization, sequence analysis, and transcription of the putative human cytomegalovirus DNA polymerase gene. *J. Virol.* **61**:119-124.
- Hermiston, T. W., C. L. Malone, and M. F. Stinski. 1990. Human cytomegalovirus immediate-early two protein region involved in negative regulation of the major immediate-early promoter. *J. Virol.* **64**:3532-3536.
- Hernandez, T. R., and I. R. Lehman. 1990. Functional interaction between the herpes simplex-1 DNA polymerase and UL42 protein. *J. Biol. Chem.* **265**:11227-11232.
- Huang, E. 1975. Human cytomegalovirus. IV. Specific inhibition of virus-induced DNA polymerase activity and viral DNA replication by phosphonoacetic acid. *J. Virol.* **16**:1560-1565.

35. Huang, E.-S. 1975. Human cytomegalovirus. III. Virus-induced DNA polymerase. *J. Virol.* **16**:298-310.
36. Jones, T. R., and V. P. Muzithras. 1992. A cluster of dispensable genes within the human cytomegalovirus genome short component: IRS1, US1 through US5, and the US6 family. *J. Virol.* **66**:2541-2546.
37. Kiehl, A., and D. I. Dorsky. 1991. Cooperation of EBV DNA polymerase and EA-D (BMRF1) in vitro and colocalization in nuclei of infected cells. *Virology* **184**:330-340.
38. Li, J., B.-S. Zhou, G. E. Dutschman, S. P. Grill, R.-S. Tan, and Y.-C. Cheng. 1987. Association of Epstein-Barr virus early antigen diffuse component and virus-specified DNA polymerase activity. *J. Virol.* **61**:2947-2949.
39. Lieberman, P. M., J. M. Hardwick, J. Sample, G. S. Hayward, and S. D. Hayward. 1990. The Zta transactivator involved in induction of lytic cycle gene expression in Epstein-Barr virus-infected lymphocytes binds to both AP-1 and ZRE sites in target promoter and enhancer regions. *J. Virol.* **64**:1143-1155.
40. Lieberman, P. M., M. Hardwick, and S. D. Hayward. 1989. Responsiveness of Epstein-Barr virus *NotI* repeat promoter to the Z transactivator is mediated in a cell-type-specific manner by independent signal regions. *J. Virol.* **63**:3040-3050.
41. Malone, C. L., D. H. Vesole, and M. F. Stinski. 1990. Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins. *J. Virol.* **64**:1498-1506.
42. Marchetti, M. E., C. A. Smith, and P. A. Schaffer. 1988. A temperature-sensitive mutation in a herpes simplex virus type 1 gene required for viral DNA synthesis maps to coordinates 0.609 through 0.614 in U_L. *J. Virol.* **62**:715-721.
43. Masse, M. J. O., S. Karlin, G. A. Schachtel, and E. S. Mocarski. 1992. Human cytomegalovirus origin of DNA replication (*ori-Lyt*) resides within a highly complex repetitive region. *Proc. Natl. Acad. Sci. USA* **89**:5246-5250.
44. Matz, B., J. H. Subak-Sharpe, and V. G. Preston. 1983. Physical mapping of temperature-sensitive mutations of herpes simplex virus type 1 using cloned restriction endonuclease fragments. *J. Gen. Virol.* **64**:2261-2270.
45. Mocarski, E. S., L. Pereira, and N. Michael. 1985. Precise localization of genes on large animal virus genomes: use of λ gt11 and monoclonal antibodies to map the gene for a cytomegalovirus protein family. *Proc. Natl. Acad. Sci. USA* **82**:1266-1270.
46. Mohr, I. J., R. Clark, S. Sun, E. J. Androphy, P. MacPherson, and M. R. Botchan. 1990. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. *Science* **250**:1694-1699.
47. Pari, G. S., and D. G. Anders. 1992. Unpublished results.
48. Pari, G. S., and S. C. St. Jeor. 1990. Human cytomegalovirus major immediate early gene product can induce SV40 DNA replication in human embryonic lung cells. *Virology* **179**:785-794.
49. Pizzorno, M. C., M. A. Mullen, Y. N. Chang, and G. S. Hayward. 1991. The functionally active IE2 immediate-early regulatory protein of human cytomegalovirus is an 80-kilodalton polypeptide that contains two distinct activator domains and a duplicated nuclear localization signal. *J. Virol.* **65**:3839-3852.
50. Purifoy, D. J. M., R. B. Lewis, and K. L. Powell. 1977. Identification of the herpes simplex virus DNA polymerase gene. *Nature (London)* **269**:621-623.
51. Purifoy, D. J. M., and K. L. Powell. 1981. Temperature-sensitive mutants in two distinct complementation groups of herpes simplex virus type 1 specify thermostable DNA polymerase. *J. Gen. Virol.* **54**:219-222.
52. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed., vol. 3. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
53. Schickendanz, J., L. Philipson, W. Ansorge, R. Pepperkok, R. Klein, and U. H. Koszinowski. 1988. The 89,000-*M_r* murine cytomegalovirus immediate-early protein stimulates *c-fos* expression and cellular DNA synthesis. *J. Virol.* **62**:3341-3347.
54. Staprans, S. I., D. K. Rabert, and D. H. Spector. 1988. Identification of sequence requirements and trans-acting functions necessary for regulated expression of a human cytomegalovirus early gene. *J. Virol.* **62**:3463-3473.
55. Stasiak, P. C., and E. S. Mocarski. 1992. Transactivation of the cytomegalovirus ICP36 gene promoter requires the α gene product TRS1 in addition to IE1 and IE2. *J. Virol.* **66**:1050-1058.
56. Stenberg, R. M., J. Fortney, S. W. Barlow, B. P. Magrane, J. A. Nelson, and P. Ghazal. 1990. Promoter-specific *trans* activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. *J. Virol.* **64**:1556-1565.
57. Tenney, D. J., and A. M. Colberg-Poley. 1991. Expression of the human cytomegalovirus UL36-38 immediate early region during permissive infection. *Virology* **182**:199-210.
58. Tenney, D. J., and A. M. Colberg-Poley. 1991. Human cytomegalovirus UL36-38 and US3 immediate-early genes: temporally regulated expression of nuclear, cytoplasmic, and polysome-associated transcripts during infection. *J. Virol.* **65**:6724-6734.
59. Wade, M., T. F. Kowalik, M. Mudryj, E. Huang, and J. C. Azizkhan. 1992. E2F mediates dihydrofolate reductase promoter activation and multiprotein complex formation in human cytomegalovirus infection. *Mol. Cell. Biol.* **12**:4364-4374.
60. Weller, S. K. 1990. Genetic analysis of HSV genes required for genome replication, p. 105-135. *In* E. Wagner (ed.), *Herpesvirus transcription and its regulation*. CRC Press, Boca Raton, Fla.
61. Weller, S. K., K. J. Lee, D. J. Sabourin, and P. A. Schaffer. 1983. Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA-binding protein. *J. Virol.* **45**:354-366.
62. Weston, K., and B. G. Barrell. 1986. Sequence of the short unique region, short repeats, and part of the long repeats of human cytomegalovirus. *J. Mol. Biol.* **192**:177-208.
63. Wu, C. A., N. J. Nelson, D. J. McGeoch, and M. D. Challberg. 1988. Identification of herpes simplex virus type 1 genes required for origin-dependent synthesis. *J. Virol.* **62**:435-443.