JOURNAL OF VIROLOGY, Nov. 1996, p. 7398–7413 Vol. 70, No. 11
0022-538X/96/$04.00+0

Evidence that the UL84 Gene Product of Human Cytomegalovirus Is Essential for Promoting *ori*Lyt-Dependent DNA Replication and Formation of Replication Compartments in Cotransfection Assays

ROBERT T. SARISKY[1] AND GARY S. HAYWARD[1,2]*

Molecular Virology Laboratories, Department of Pharmacology and Molecular Sciences,[1] and Department of Oncology,[2] Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185

Received 16 April 1996/Accepted 23 July 1996

The protein products of 11 viral genomic loci cooperate in a transient cotransfection assay to mediate lytic-phase DNA replication of *ori*Lyt, the human cytomegalovirus (HCMV) origin of replication. Six of these genes have homology with the well-characterized herpes simplex virus replication genes and encode core replication machinery proteins that are typically essential for DNA synthesis. The remaining five HCMV gene loci, initially referred to as auxiliary components, include several known immediate-early (IE) transcriptional regulatory proteins as well as genes encoding functionally uncharacterized polypeptides. Some or all of the auxiliary components may be necessary in *trans* to replicate the HCMV *ori*Lyt only because they are required for efficient expression or transactivation of the native early promoters and 3′ processing elements included in the genomic clones. Therefore, we reassessed the requirements for the auxiliary components by adding constitutive heterologous promoters and control signals to the coding regions and carrying out transient *Dpn*I replication assays in cotransfected Vero cells. The results revealed that in the presence of the UL69 posttranscriptional activator and the remaining auxiliary polypeptides, UL84 was the only auxiliary component that could not be omitted to obtain *ori*Lyt-dependent DNA replication. Nevertheless, in human diploid fibroblasts, some additional auxiliary loci as well as UL84 were critical. There was also an obligatory requirement for UL84, in cooperation with two other auxiliary factors, UL112-113 and IE2, and the core machinery, to constitute the minimal HCMV proteins necessary to direct *ori*Lyt-dependent DNA amplification. However, the Epstein-Barr virus core replication genes could substitute for the HCMV core genes, and in these circumstances, UL84 alone directed amplification of HCMV *ori*Lyt. Moreover, there was also an absolute requirement for UL84 along with the core and other auxiliary factors for the formation of intranuclear replication compartments as assayed by immunofluorescence in transient DNA cotransfection assays. These compartments were typical of those associated with active viral DNA replication in HCMV-infected cells, they incorporated pulse-labeled bromodeoxyuridine, and their formation was both phosphonoacetic acid sensitive and *ori*Lyt dependent. These results demonstrate that UL84 is obligatory for both intranuclear replication compartment formation and origin-dependent DNA amplification and suggest that it is a key viral component in promoting the initiation of HCMV *ori*Lyt-directed DNA replication.

Genetically defined *cis*-acting elements which function as origins of DNA replication frequently comprise both a core component, absolutely required for replication, and one or more auxiliary components, often composed of promoter and enhancer elements that are dispensable for replication (21–23). Initiation typically begins within the central core region adjacent to an adenine-thymidine (AT)-rich motif capable of being easily unwound, while the auxiliary elements may determine host range specificity or replication efficiency (11, 18, 21, 22, 36, 41, 46, 90). The replicon model postulates that in order for DNA synthesis to begin, a sequence-specific recognition event by an initiator protein is typically required. In higher eukaryotes, identification of such initiator elements and replicator sequences remains behind the success garnered by the study of simpler viral systems (11, 28, 68) and yeast cells (32, 61, 63, 76). In most viruses studied to date, the core origin region is bound by a virus-specified origin-binding protein that recruits the core replication machinery and often also possesses helicase or unwinding activity itself (7, 17, 89). For example, these functions are performed in simian virus 40 (SV40) by the large T antigen (6, 11, 81, 82, 84), in herpes simplex virus type 1 (HSV-1) by UL9 (28, 55), and in papillomavirus by the E1 polypeptide (53). In Epstein-Barr virus (EBV), the transactivator Zta performs some of these functions, but until now, a replication-initiating or -promoting activity encoded by a human cytomegalovirus (HCMV) polypeptide has not been identified.

HCMV provides a complex eukaryotic replication system which remains mechanistically uncharacterized. This human betaherpesvirus is an important pathogen which manifests itself clinically by causing congenital birth defects (74), and perhaps coronary restenosis (73), as well as life-threatening infections in immunocompromised individuals, including recipients of organ transplants (27, 33), persons suffering from AIDS (3), or those receiving chemotherapy (29, 40). The HCMV genome is a 229-kb linear double-stranded DNA molecule which potentially encodes approximately 200 genes (12, 78). After entry into permissive cells and viral uncoating, the linear genome is thought to circularize and then to replicate by

* Corresponding author. Mailing address: Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205-2185. Phone: (410) 955-8684. Fax: (410) 955-8685.

TABLE 1. HCMV replication genes and expression plasmids

HCMV(AD169) gene	Predicted function	Clone[a]	Nucleotide position[b]	
			ATG	Stop
Core machinery				
UL44	Polymerase accessory	pRTS22	56512	55214
UL54	DNA polymerase	pRTS6	80631	76905
UL57	Single-stranded DNA-binding protein	pRTS7	90281	86577
UL70	Primase	pRTS9	103373	100537
UL102	Primase-associated factor	pRTS29	146520	149140
UL105	DNA helicase	pRTS10	151850	154793
Auxiliary components				
UL36-38	Regulatory	p302	52706	48246
IRS1	Regulatory	pRTS18	189765	192302
IE1/IE2 (UL122/3)	Regulatory	pRL45	172765	169367
UL112-113	Unknown	pRTS26	160589	162797
UL84	Unknown	pRTS5	123070	121312
Stimulatory factor				
UL69	Posttranscriptional, regulatory	pRTS28	100433	98202

[a] p302 and pRL45 two encode multiple proteins and mRNA that are jointly controlled by the HCMV IE promoter and their natural context polyadenylation processing signals.

[b] Genomic locations of the translation start and stop sites for the individual open reading frames of the HCMV replication loci were subcloned into an expression vector (pRTS2) containing SV40 enhancer, β-globin intron, and SV40 polyadenylation signals as described in Materials and Methods.

a process which produces concatemeric molecules that are subsequently cleaved into unit-length genomes and packaged (47, 52). The *cis*-acting origin of HCMV lytic DNA replication (*ori*Lyt) was originally mapped to a single precise location within the viral genome by a chain termination approach (37), and bacterial plasmids carrying this sequence have been shown to replicate after introduction into cells by a transient transfection assay in which the viral replication machinery was supplied in *trans* by virus infection (1, 2, 51, 56).

Although the overall replication scheme appears similar to its counterpart in alphaherpesviruses (62), the lytic replication process of HCMV is likely to be more complex for several reasons: (i) HCMV *ori*Lyt spans more than 2.4 kb (1); (ii) *ori*Lyt contains numerous reiterated sequence elements (both known consensus transcription factor recognition sites and numerous uncharacterized motifs) (1); (iii) deletion analyses within *ori*Lyt suggest that little of the 2.4-kb sequence is dispensable for function (1); (iv) *ori*Lyt function requires 11 distinct gene loci which mediate DNA replication in a transient assay (56); (v) HCMV infection stimulates expression of cellular S-phase and growth genes (4, 5, 15, 27, 43, 66, 77, 94), whereas most viral systems terminate cellular functions (62); and (vi) maximal viral DNA synthesis occurs approximately 72 h postinfection (hpi), indicating a significant delay not shared with the alphaherpesviruses (62). This situation is in striking contrast to the viral origins of HSV and SV40 in particular, which simply include a small AT-rich region and a core component of specific-sequence reiterations for a single viral origin-binding protein required to initiate DNA synthesis, as well as one or more accessory *cis*-acting components (11, 21, 22). Despite organizational similarities among the lytic viral origins of replication of SV40, HSV, EBV, and adenovirus, the HCMV origin remains distinguishable by its apparently greater complexity and resemblance to putative mammalian cellular origins such as the extensive region downstream of the dihydrofolate reductase gene in Chinese hamster ovary cells. Interestingly, it is thought that rather than utilizing unique essential sequences to initiate replication as found for simple viral systems, mammalian cells may use dispersed, redundant *cis*-acting elements, a feature possibly shared with HCMV initiation (24, 25).

Recently, Pari and Anders (56) have identified 11 distinct viral loci encoding *trans*-acting factors sufficient for transient complementation of *ori*Lyt-dependent DNA replication in human diploid fibroblast (HF) cells (56). The previous similar exploitation of such a transient expression system significantly contributed to the identification of all seven essential virus-encoded genes required for origin-specific DNA replication of HSV-1 (10, 93) and later for EBV also (30). Six of the essential HCMV loci span homologs or probable homologs of the HSV-1 (30, 85) and EBV core replication genes (30), encoding the DNA polymerase (UL54), the single-stranded DNA-binding protein (UL57), a putative helicase-primase complex (UL70, UL102, and UL105), and a polymerase-associated factor (UL44) (56). These six polypeptides comprise the core replication machinery and are outlined in Table 1. A requirement for an additional five HCMV loci, initially termed auxiliary factors, was established in the transient assay. These included known transactivators of gene expression, including UL36-38, IRS1, and the major immediate-early (IE) region (UL122-123) encoding both IE1 and IE2, as well as two novel loci, which had not previously been implicated in either gene regulation or viral replication, i.e., UL84 (38, 65, 72) and UL112-113 (91, 92).

One limitation of the previous form of this assay for HCMV was the use of viral components regulated by their native promoter elements and 3′ untranslated control regions, which leads to the inability to define directly the minimal essential components for replication. For example, in the case of HSV-1, seven distinct HSV early genes encoding the six core polypeptides, and the UL9 origin-binding protein plus two IE genes, were required to mediate HSV origin-specific DNA replication when introduced along with target origin plasmid DNA into mammalian cells by transient transfection (93). When those same essential *trans*-acting factors were expressed from a heterologous constitutive promoter, the minimal components required solely for origin-directed DNA amplification were identified, indicating that the IE proteins were dispensable and thus not directly needed for DNA replication itself (39). Most likely, they were critical for the transactivation of the homologous viral early promoters, as well as posttranscriptional activity, to permit efficient expression of the replication

gene products (83, 93). Therefore, their involvement in the initiation of DNA replication remained indirect and they were dispensable for the formation of a replication fork initiation complex. In this report, we have undertaken a systematic approach with the HCMV replication machinery to eliminate factors that are required only for efficient gene expression purposes and to address which, if any, viral components are directly essential for the initiation process.

The Challberg-designed transient DNA cotransfection replication assay additionally provides a powerful tool for the identification of which viral factor serves as a specific origin-binding or targeting protein that mediates the initiation of replication. When this assay approach (10, 93) was applied for replication of EBV *ori*Lyt in Vero cells, it resulted in the identification of six core replication proteins, as well as three IE transactivators, Zta, Rta, and Mta (30, 31). Subsequently, both Rta and Mta were found to be dispensable when the core components were placed into heterologous SV40 enhancer-driven expression vectors (31, 67). Although attempts to replace individual EBV replication genes by their HSV-1 counterparts proved futile, a group substitution approach aided in resolving which EBV replication gene(s) provided the essential origin-binding function. The complete set of HSV-1 core replication genes (minus UL9) faithfully replicated EBV *ori*Lyt in a transient transfection assay but did so only when additionally supplied with a Zta expression plasmid, thus identifying Zta as the only EBV polypeptide that is indispensable for EBV-specific *ori*Lyt replication (31). Mutations of the Zta binding sites within *ori*Lyt confirmed these findings (68, 69). In this study, we have performed similar substitution experiments using the HCMV and EBV core replication systems in a successful attempt to identify an apparent origin-specific initiator protein encoded by HCMV.

In addition, we have approached the identification of a viral replication-promoting factor(s) by another criterion, that of the formation of intranuclear structures termed viral replication compartments (19, 20, 34, 50, 54, 60, 87). Viral infection, including that of herpesviruses, typically results in the formation of large globular amorphous replication compartments (perhaps derived from cellular replisomes) consisting of progeny DNA and the viral replication machinery, which can be visualized either directly by phase microscopy, by viral DNA in situ hybridization, or by immunofluorescence with antibody against core replication proteins such as the viral single-stranded DNA-binding protein (9, 87). This report represents the first demonstration that herpesvirus replication compartments can be generated by cotransfection, as detected by indirect immunofluorescence assay (IFA), and that this can be accomplished with just the viral replication-associated genes and the origin sequences in the absence of virus infection. Our system is absolutely dependent on the presence of target origin DNA sequences, the core replication machinery proteins, and at least one additional polypeptide serving an essential origin function, UL84, although other auxiliary replication proteins such as UL112-113 and IE2 may also contribute directly to the formation of a replication fork complex and to the overall efficiency of the system. Both our IFA and our transient transfection replication assay data taken together suggest that UL84 is a key viral factor responsible for promoting HCMV *ori*Lyt-directed DNA replication.

MATERIALS AND METHODS

Cells and virus. Vero (African green monkey kidney) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. HF cells at passage 9 were maintained in DMEM containing 10% fetal bovine serum. The AD169 strain of HCMV [HCMV(AD169)] was a gift from D. J. Spector (Pennsylvania State University, Hershey). Stocks of HCMV were prepared by infecting monolayers of HF cells with 0.01 PFU per cell. After viral adsorption in serum-free medium for 1 h at 37°C, the cells were maintained with DMEM supplemented with 5% fetal bovine serum and placed at 37°C in a humidified 5% CO_2 incubator. Medium was changed after 4 days. When 75% of the cells demonstrated cytopathic effects, the volume of medium was reduced by half. Two days later, the cells and extracellular medium were collected, subjected to several freeze-thaw cycles, and centrifuged to pellet cell debris. Virus was stored at −80°C and, after titer was determined by plaque formation on HF cells, used directly for infections.

Replication machinery expression plasmids. HCMV(AD169)-infected cell DNA was purified as described previously (47) to serve as a DNA template source from which to clone the viral replication genes. The EBV parent clones containing the replication genes from which second-generation expression vectors were constructed were described previously (67). Synthetic oligonucleotide primers (JHU Core Facility) were used to amplify by PCR the coding regions of viral replication genes extending from the ATG translational start site to the translational stop codon as indicated in Table 1. A modified pSG5 vector (Stratagene) was constructed by destroying the *Xba*I site and then placing a pUC18-plus-*Bgl*II polylinker from pGH57 into the *Eco*RI-*Bgl*II site to create plasmid pRTS2. After amplification of each of the appropriate viral open reading frames from virion DNA, the products were cleaved with zero-cut boundary restriction endonucleases and subcloned into pRTS2 as follows: pRTS5, *Xba*I-*Pst*I; pRTS6, -7, -9, -11 to -16, -25, -26, and -28, *Xba*I-*Hin*dIII; pRTS10, *Bgl*II; pRTS18, -21, and -29, *Bam*HI-*Bgl*II; and pRTS22, *Bam*HI-*Sal*I. Plasmids pRL45, encoding IE1 and IE2, and p302 (a gift of A. Colberg-Poley, Children's National Medical Center, Washington, D.C.), encoding UL36-38 polypeptides, are regulated by the HCMV major IE promoter and were described previously (59, 79). Additionally, the UL69 open reading frame was amplified from HCMV(Towne)-infected cell DNA by oligonucleotide-directed PCR extending from the genomic nucleotide 100433 (ATG) to 98202 (stop) and subcloned into pRTS2 as an *Xba*I-*Hin*dIII fragment to create pRTS28.

Transient transfection replication assay. Vero and HF cells were transfected by the method of Chen and Okayama as described by Pizzorno et al. (59) and Pari and Anders (56), respectively. Approximately 10 μg of total DNA containing the replication plasmids listed in Table 1 was cotransfected with 4.0 μg of viral origin DNA (either pSP50 or pSP38) into 10^6 cells plated onto a 100-mm-diameter dish. The cells were harvested 80 h after the posttransfection medium change by first washing the monolayer twice with phosphate-buffered saline (PBS) and subsequently scraping it into 4 ml of 40 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA–150 mM NaCl. The cells were pelleted and lysed in 2 ml of lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 2% sodium dodecyl sulfate [SDS], 100 μg of proteinase K per ml). Following overnight incubation at 37°C, the samples were diluted to 4 ml with Tris-EDTA (pH 8.0), extracted with phenol, phenol-chloroform, and chloroform, and ethanol precipitated upon the addition of sodium acetate (pH 5.2) to a final concentration of 0.3 M. The DNA pellets were resuspended in 450 μl of distilled H_2O (dH_2O), treated with 100 μg of RNase A per ml, ethanol precipitated, and resuspended in 300 μl of dH_2O. Approximately 10 μg of transfected cell DNA was digested with 30 U either *Kpn*I or *Xba*I in a 100-μl reaction volume for 24 h at 37°C. The samples were then ethanol precipitated, resuspended in 50 μl dH_2O, and digested with 30 U of *Dpn*I overnight at 37°C. To monitor the *Dpn*I activity, 5 μl of the *Dpn*I reaction digest was removed and incubated simultaneously with 500 ng of pUC19 DNA overnight at 37°C. Complete cleavage of the pUC19 DNA indicated that the experimental DNA was also completely digested. The cellular DNA was resolved by electrophoresis on a 0.8% agarose gel at 35 V for 20 h, transferred to either a Nytran membrane (Schleicher and Schuell) or a nylon Zeta-probe membrane (Bio-Rad) after treatment of the gel at 20°C for 10 min in 200 mM HCl (used only for blots analyzing *Xba*I-digested samples because of the size of the origin-containing fragment), and then incubated in 0.4 M NaOH–0.6 M NaCl for 20 min. The gel was then transferred by vacuum transfer to the nylon membrane in the presence of 10× SSC (1.5 M NaCl, 0.15 M sodium citrate) for 30 min. After the membrane was dried completely at 20°C, the DNA was irreversibly cross-linked by UV radiation onto the nylon. The membrane was prehybridized for 2 to 4 h at 60°C in 25 ml of buffer consisting of 1% SDS, 5 mg of Carnation nonfat dried milk per ml, 0.5 mg of heparin per ml, 0.2 mg of denatured salmon sperm DNA per ml, 60 mg of polyethylene glycol 8000 per ml, 5× SSPE (750 mM NaCl, 50 mM Na_2HPO_4, 5 mM Na_2EDTA), and 10% formamide. Approximately 50 ng of a gel-purified *Kpn*I *ori*Lyt fragment was radiolabeled with [α-^{32}P]dATP by random priming to a specific activity of 10^8 cpm/μg as instructed by the manufacturer (Boehringer Mannheim). The membrane was then incubated overnight at 60°C with 10^6 cpm of denatured radiolabeled *ori*Lyt probe DNA per ml and fresh prehybridization buffer. Following hybridization, the membrane was washed twice in 0.1× SSC–0.1% SDS at 65°C for 45 min and exposed to Kodak XAR5 film for 24 h at −80°C, using an intensifying screen. The resulting autoradiograms were quantified with a Kontes Fiber Optic Scanner.

IFA. HF or Vero cells were plated at approximately 0.8×10^5 cells per well onto glass tissue culture chamber slides (Nunc). For infection, HCMV(AD169) was added to monolayers of HF cells at a multiplicity of infection of approximately 5 PFU per cell. DNA transfections were done as described above, with the following modification: a total of 1.5 μg of DNA was transfected per well, using 100 ng of each plasmid. Some samples as indicated were incubated with

phosphonoacetic acid (PAA) at 400 μg/ml for the duration of experiment. At 72 h after the posttransfection medium change, the cells were fixed in 2% paraformaldehyde in PBS for 10 min at 20°C and then permeabilized in 0.2% Triton X-100 in PBS for 20 min at 4°C. After being rinsed in 1× Tris-saline, the slides were incubated for 1 h at 37°C in a humidified tray with 2% goat serum and a 1:2,000 dilution of anti-HCMV UL44 mouse monoclonal antibody (MAb) (Advanced Biotechnologies Inc.). After incubation, the slides were washed for 15 min in 1× Tris-saline at 20°C and incubated with a 1:100 dilution of rhodamine-conjugated goat anti-mouse immunoglobulin antibody (Cappel) for 30 min at 37°C. Prior to the addition of mounting solution and a coverslip, the slides were once again washed for 15 min in 1× Tris-saline at 20°C. Slides were screened and photographed with a 40× oil immersion objective on a Leitz Dialux 20EB epifluorescence microscope, using Kodak T-MAX 3200 film.

Transfected Vero cells pulse-labeled with 10 μM bromodeoxyuridine (BUdR) for 20 min at 37°C were fixed and permeabilized as described above. The cells were then treated with 4 N HCl for 10 min at 20°C to expose the incorporated BUdR residues and washed twice for 5 min each with PBS to neutralize the acid. The HCl treatment procedure did not affect the ability of anti-UL44 to recognize its target antigen. The slides were then incubated either with the anti-UL44 mouse MAb mentioned above or with a 1:125 dilution of anti-BUdR mouse MAb (Becton Dickinson) with 2% goat serum for 1 h at 37°C in a humidified tray. The slides were washed, incubated with secondary antibody, washed, and mounted as described above.

RESULTS

Identification of individual HCMV auxiliary components dispensable for *ori*Lyt-dependent DNA synthesis in cotransfection assays in Vero cells. Candidate replication genes were subcloned into a modified pSG5 vector, placing them under the control of the SV40 early promoter and polyadenylation region with addition of a 5′ β-globin splice signal to stabilize the mRNA (Table 1). Thus, by removing the viral genes from their native promoters and 3′ processing elements, we hoped to distinguish which individual gene products are directly required for DNA replication and effectively eliminate those viral factors that simply function in a transcriptional or post-transcriptional capacity to activate expression of the *trans*-acting genes. The plasmids described in Table 1 were transfected into Vero or HF cells along with a target HCMV origin for their analysis in the transient transfection replication assay. Two separate target plasmids containing origin fragments of different sizes, pSP38 and pSP50 (1), were used in this study. The former represents a 5.9-kb origin fragment extending from HCMV genomic positions 89795 to 95671, whereas the latter contains a 5.1-kb subfragment ranging from 89795 to 94860. The transient replication assay measures amplification by exploiting the lack of methylation and subsequent loss of *Dpn*I restriction enzyme sensitivity of progeny plasmid DNA sequences that have been newly synthesized in mammalian cells. Only those target DNAs with origins that undergo amplification upon transfection will become resistant to cleavage by *Dpn*I and can be detected by Southern blot hybridization. All input plasmid molecules unable to replicate in vivo will remain methylated and be cleaved by the restriction enzyme.

HCMV fails to synthesize progeny viral DNA in most transformed cells, including the Vero cell line; however, the restrictive block is targeted at the IE mRNA level and not necessarily at viral DNA synthesis (47). We have successfully cotransfected Vero cells with the complete set of 11 replication plasmids outlined in Table 1 plus an HCMV target *ori*Lyt plasmid to produce *Dpn*I-resistant replicated progeny DNA in a transient assay. A portion of the total cellular DNA isolated at 80 h posttransfection was first cleaved with either *Xba*I (which linearizes the *ori*Lyt plasmids at approximately 9.0 kb) or *Kpn*I (which separates the approximately 6.0-kb origin fragment from the 3.0-kb vector DNA) and next treated with *Dpn*I to assay for DNA amplification in mammalian cells. The core replication machinery alone, which represents the six homologs of HSV-1 replication genes, is insufficient to initiate

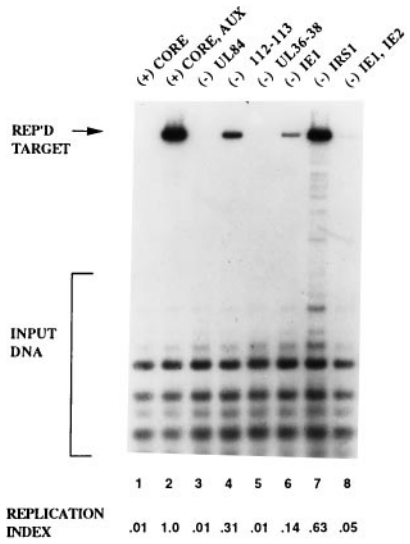

FIG. 1. HCMV *ori*Lyt DNA replication by constitutively expressed core machinery proteins depends on only UL84 and UL36-38, individually, in the presence of the remaining 10 viral components in cotransfection omission replication assays in Vero cells. Shown are results of a transient replication assay using the viral origin template pSP38. Vero cells were cotransfected with the six core replication expression plasmids, the *ori*Lyt target pSP38, and the five auxiliary (AUX) replication expression plasmids minus the one component listed above each lane. Isolated cell DNA was digested with *Xba*I and *Dpn*I, and a Southern blot of the separated DNA fragments was hybridized with an isolated ^{32}P-labeled *Kpn*I fragment of *ori*Lyt as the probe DNA. Replicated *Dpn*I-resistant pSP38 and unreplicated input DNA are marked REP'D TARGET and INPUT DNA, respectively. The RI value shown below each lane corresponds to the ratio of the *Dpn*I-resistant band relative to the first *Dpn*I-sensitive (input DNA) band. This value was set at 1.0 for the positive control containing all components shown in lane 2.

and subsequently mediate DNA replication (Fig. 1, lane 1). The absence of a full-length band on the autoradiogram indicated that the *Dpn*I restriction digestion was performed to completion. Only upon supplementation of the core replication machinery with the five auxiliary components did authentic *ori*Lyt-dependent DNA synthesis occur (lane 2). The addition of PAA (400 μg/ml) to the complete replication assay abolished all detectable replication (not shown).

Next, we performed an omission assay to identify those auxiliary components absolutely required for *ori*Lyt replication. In the omission assay, a single auxiliary component is removed from the transfection mixture of target origin, core replication machinery expression plasmids, and the remaining auxiliary expression plasmids. Using pSP38 as the target origin, we were able to identify a strict requirement for both UL84 (Fig. 1, lane 3) and UL36-38 (lane 4), since replication was abolished in the absence of either of these two components. Furthermore, the UL112-113 locus (lane 5) and IRS1 (lane 6), as well as IE1 plus IE2 (lane 7), were all dispensable for replication activity, although UL112-113 and IE2 were critical to obtain optimal levels of replication.

Cross-comparisons between replication assays from different transfection experiments cannot be accurately assessed. However, a replication index (RI) value was calculated to provide a general indication of the relative replication efficiencies of different samples within individual transfection experiments. This value corresponds to the ratio of the densitometric quantitation of the *Dpn*I-resistant (replicated DNA) band relative to the first *Dpn*I-sensitive (input unreplicated DNA) band. An RI of 1.0 was established for the optimal condition whereby all 11 viral (core and auxiliary) replication machinery components

were included in the assay. Results for samples within the same transfection experiment were then compared with the preset value of 1.0, and the resulting RI values are given below the lanes in Fig. 1. The RI for the omission of UL84 or UL36-38, which failed to result in a detectable amplified origin product, was determined to be 0.01. Exclusion of the UL112-113 expression plasmid from this experiment reduced the replication level by approximately three-fold (RI of 0.31), whereas omission of IRS1 had a minimal effect, as indicated by the quantitative RI of 0.63. Omission of IE1 and IE2 from the transient assay resulted in a 20-fold decrease in amplification (RI of 0.05) compared with the presence of all 11 viral loci (Fig. 1, lane 2). Also, the impaired replication activity associated with the collective loss of IE1 and IE2 was shown to result from an independent activity associated with each polypeptide (lanes 6 and 8). This drop in the level of replication is not necessarily unexpected, given that IE1 and IE2 may have synergistically increased expression from our SV40 early promoter-driven plasmids (59, 71, 75, 80). Nonetheless, our results suggest that only UL84 and UL36-38 and none of the latter three genetic loci are absolutely required for the formation of a replication initiation complex when the remaining auxiliary components are present.

We have reproduced the results of the transient omission assay by using a second target origin, pSP50, and identified the same two auxiliary components (i.e., UL84 and UL36-38) as being essential for replication. The RI values for the removal of these two loci from the replication assay were calculated to be 0.08 and 0.07, respectively. Again, each of the other three auxiliary polypeptides omitted one at a time remained nonessential in the presence of the other four auxiliary factors (data not shown). Omission of IRS1 generally had a minimal effect on the efficiency of *ori*Lyt replication (RI of 0.93), although UL112-113 and IE1/IE2 made significant contributions. For example, the loss of IE1/IE2 from the replication assay using the pSP50 target origin DNA decreased the replication level 6-fold (RI of 0.16) below that of the wild type, whereas the RI for transient DNA synthesis in the absence of UL112-113 was 0.43 in the same experiment but varied from a 3- to 10-fold reduction within several other experiments.

Loss of the UL36-38 dependence, but not UL84 dependence, for *ori*Lyt DNA replication in an omission assay in Vero cells in the presence of UL69. The UL36-38 genomic locus represents a complex alternatively spliced transcription unit that encodes three IE transcripts as well as an additional abundant early class mRNAs (45, 86). The gene products from this region can act to regulate expression of both viral and cellular promoters in a host- and promoter-dependent manner (16). Their requirement for the lytic cycle in vivo has been established by using an antisense phosphorothioate oligonucleotide complementary to the intron-exon boundary of the UL36 and UL37 unspliced mRNAs (16, 57, 70). Our initial transient assay yielded an apparently similar requirement for this region in the DNA replication assay (Fig. 1).

The UL69 open reading frame of HCMV, the product of which is homologous to the IE proteins ICP27 of HSV-1 (12) and Mta of EBV (14, 49), encodes at least two differentially regulated transcripts that are detected as early as 7 hpi. Several studies have demonstrated the ability of ICP27 to stimulate and regulate HSV-1 DNA synthesis (64, 93), and Uprichard and Knipe (83) noted increased expression of certain HSV-1 DNA replication proteins in the presence of this polypeptide. The equivalent gene product from EBV, encoded by BSLF2/BMLF1 (Mta), primarily serves a posttranscriptional function to indirectly contribute to EBV replication efficiency (8, 44). UL69 localizes within the nuclei of both transfected and virus-infected cells and can stimulate expression from an HCMV early promoter as well as several heterologous promoters (88). A posttranscriptional mechanism of action such as mRNA stabilization has been proposed for UL69 to account for the broad activation observed in transient reporter assays (88).

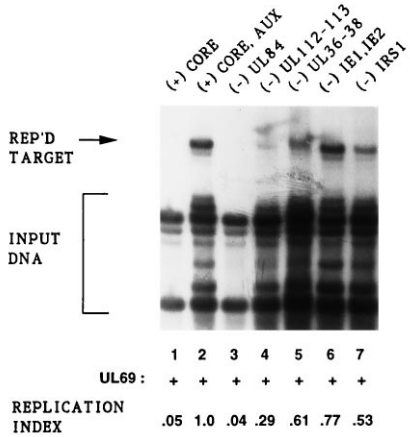

FIG. 2. HCMV *ori*Lyt DNA replication by the viral core machinery proteins depends only on UL84 in the presence of UL69, the core replication machinery, and the remaining auxiliary factors in cotransfection replication assays in Vero cells. Vero cells were cotransfected with the six core replication expression plasmids, pSP50 *ori*Lyt, and the five auxiliary (AUX) replication expression plasmids minus the one component listed above each lane. Southern blot analysis of the transient replication assay was performed as described for Fig. 1 except that UL69 encoded by pRTS28 was added to the experimental lanes as indicated. The transfected cell DNA was cleaved with *Kpn*I and *Dpn*I. The replicated and unreplicated DNA are denoted by REP'D TARGET and INPUT DNA, respectively, and the RI values are indicated at the bottom.

With the intent of increasing the level of HCMV *ori*Lyt replication, in a manner equivalent to Mta stimulation of EBV replication, we supplemented our transient replication assay with an SV40 enhancer-regulated UL69 expression plasmid. Surprisingly, under these conditions, the requirement for the UL36-38 products was abolished (Fig. 2, lane 5). In contrast, the requirement for UL84 was not affected (lane 3). This result distinguishes UL84 as the only auxiliary component absolutely essential for origin amplification in the presence of the core and four remaining auxiliary factors. The mechanism by which the UL69 gene product compensates for the replication function contributed by UL36-38 is unknown; however, they most likely share an ancillary function. In addition, UL69 also greatly enhanced replication function in the absence of IE1 and IE2, further implicating the ability to potentially interchange some transactivation activities (lane 6). However, the threefold reduction in replication activity associated with the absence of the UL112-113 locus was not enhanced by the presence of UL69 (lane 4).

Curiously, the UL69 gene product was not selected in the original transient complementation assay used by Pari and Anders (56), which identified 11 viral loci as essential for replication, and it was not essential in our assay either. Therefore, it cannot itself serve a critical function in the initiation process. The discovery that the otherwise essential requirement for UL36-38, even with SV40 enhancer-driven replication genes, can be substituted with a UL69 activity leads us to speculate that the most likely role associated with UL36-38 is to stimulate posttranscriptional events. Evidently, UL84 represents the only virus-encoded auxiliary component absolutely required for DNA synthesis when replication conditions are optimal, i.e., in the presence of the core and all other auxiliary

TABLE 2. EBV replication genes and expression plasmids

Viral gene	Predicted function	Clone
Core machinery		
BMRF1	Polymerase accessory	pRTS14[a]
BALF5	DNA polymerase	pRTS13
BALF2	Single-stranded DNA-binding protein	pRTS12
BSLF1	Primase	pRTS11
BBLF2/3	Primase-associated factor	pRTS25
BBLF4	DNA helicase	pRTS28
Auxiliary components		
BMLF1 (Mta)	Regulatory, posttranscriptional	pRTS16
BRLF1 (Rta)	Regulatory	pRTS15
Initiator protein		
BZLF1 (Zta)	Regulatory, origin-binding protein	pRTS21

[a] The open reading frames of the EBV replication loci were subcloned into an expression vector (pRTS2) containing the SV40 enhancer, β-globin intron, and SV40 polyadenylation signals as described in Materials and Methods.

replication factors. Some of the other auxiliary replication proteins, especially UL112-113, do, however, significantly contribute to the overall efficiency of the system. Therefore, our data suggest that UL84 is the best candidate to fulfill an initiator-specific function.

A heterologous viral replication core machinery substitution approach to identify an initiator(s) of HCMV DNA replication in cotransfection assays in Vero cells. Mammalian herpesvirus genomes all encode six core replication machinery genes which exhibit either limited or extensive amino acid sequence homology among the three viral classes; however, in general, origin-binding protein homologs have not been identified in the different herpesviruses. The EBV initiator of replication, Zta, possesses neither positional nor sequence relationships with the HSV-1 UL9 origin-binding protein (30, 31). However, we and others demonstrated previously that the core replication machinery from one viral class can substitute for the components of another viral class to mediate DNA replication in the presence of the appropriate specific initiator polypeptide and *cis*-acting origin sequences (31, 39). To extend this approach, we attempted to search for initiator properties possessed by one or more of the HCMV auxiliary components when cotransfected with the EBV core machinery and the HCMV origin template. The EBV core replication machinery listed in Table 2 alone, as expected, proved incapable of directing transient DNA replication of the HCMV *ori*Lyt (Fig. 3A, lane 1). Additionally, the same EBV core components, even when supplemented with the three EBV transactivators Zta, Rta, and Mta, were unable to mediate HCMV DNA synthesis (lane 2). Authentic HCMV origin-dependent DNA synthesis ensued only upon the complementation of the EBV core machinery with the five HCMV auxiliary components, including UL84 (lane 3). The implication is that one or more of the HCMV auxiliary components can execute specific initiator activities.

Therefore, we set out to identify which polypeptides can mediate this function by using the heterologous viral complementation assay. The EBV core replication machinery (Fig. 3B, lane 1) was supplemented with UL84 only, with the UL36-38 loci alone, with UL69 alone, with UL84 plus UL36-38, or with UL84 plus UL69 and examined for the ability to replicate the HCMV *ori*Lyt. Cotransfection of the EBV core replication machinery expression plasmids with only a single HCMV expression plasmid, that encoding UL84, proved to be sufficient to direct HCMV *ori*Lyt DNA synthesis (lane 2). The addition of either UL36-38 or UL69 to the assay further enhanced the replication level in the presence of UL84 (lanes 5 and 6); however, they were clearly not obligatory for replication. Surprisingly, the absolute requirement for either UL36-38 or UL69 was dispensed with when the EBV core replication expression plasmids were used, unlike in the presence of the HCMV core replication expression plasmids (Fig. 1 and 2). These results indicate that UL84 alone, in combination with the EBV core protein machinery, is capable of functioning to promote HCMV *ori*Lyt-dependent DNA replication in transient replication assays in Vero cells.

Identification of the minimal HCMV auxiliary components necessary in conjunction with the core replication machinery to detect *ori*Lyt DNA synthesis in cotransfection assays in Vero cells. Once we established that UL84 is absolutely required for *ori*Lyt DNA synthesis in a transient cotransfection assay which included all other auxiliary components, we set out to determine the minimal HCMV auxiliary loci necessary to detect *ori*Lyt replication at the level of Southern blot analysis. We demonstrated in the experiments shown in Fig. 1 and 2 that depletion of IRS1 or IE1 activity from the replication assay failed to significantly affect the replication efficiency; however, the individual loss of UL112-113, IE2, or UL36-38 was more critical. Therefore, we performed mix-and-match experiments using UL84, UL112-113, IE2, and UL36-38 to identify whether any combination(s) of only two or three of these four auxiliary components are minimally capable of mediating *ori*Lyt DNA synthesis in cooperation with the core replication machinery in the presence of UL69.

The results revealed that no two auxiliary components alone, including UL84 plus UL69, were sufficient to result in the amplification of *ori*Lyt, thus demonstrating that the EBV and HCMV core sets are not completely equivalent in substitution assays (Fig. 4, lane 1). However, the combination of UL84, UL112-113, and IE2 provided the minimal components required to replicate *ori*Lyt in combination with the core replication machinery proteins plus UL69 (lane 3). Both the UL112-113 and IE2 components were necessary for replica-

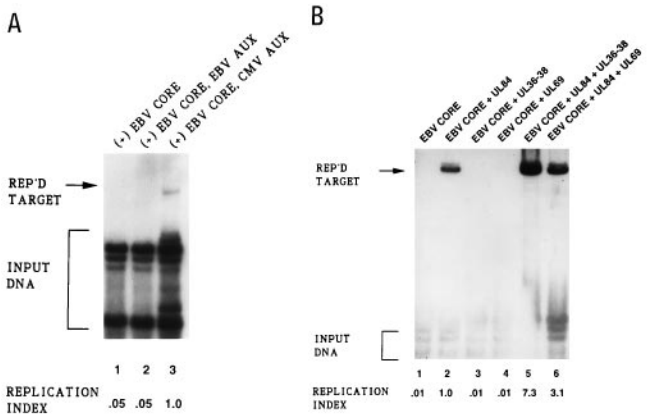

FIG. 3. Complementation of HCMV *ori*Lyt replication with the EBV core replication machinery proteins and UL84 in Vero cells. (A) Vero cells were cotransfected with the six EBV core replication expression plasmids, pSP50 (containing the HCMV target *ori*Lyt), and either the three EBV auxiliary (AUX) components or the entire set of five HCMV auxiliary expression plasmids (minus UL69) as indicated above each lane. The transfected cell DNA was cleaved with *Kpn*I and *Dpn*I, and Southern blot analysis was performed as described for Fig. 1. (B) Vero cells were cotransfected with the EBV core replication expression plasmids, pSP38, and the HCMV auxiliary expression plasmid indicated above each lane. Southern blot analysis of the *Xba*I- and *Dpn*I-digested transient replication assay was performed as described for Fig. 1. The replicated and unreplicated DNA are denoted by REP'D TARGET and INPUT DNA, respectively, and the RI values are indicated at the bottom.

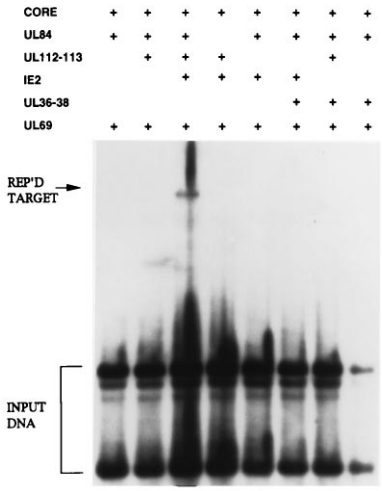

FIG. 4. Identification of the minimal auxiliary factors required to direct *ori*Lyt DNA synthesis in cotransfection assays. Vero cells were cotransfected with the HCMV core replication expression plasmids, pSP50 target origin, and the combinations of auxiliary components as indicated at the top by a plus sign. The transfected cell DNA was cleaved with *Kpn*I and *Dpn*I. The replicated and unreplicated DNAs are denoted by REP'D TARGET and INPUT DNA, respectively.

tion, since either alone in addition to UL84 and UL69 could not mediate *ori*Lyt DNA replication in cooperation with the HCMV core protein replication machinery (lanes 2 and 5). Additionally, supplementation of the core components and UL69 with UL112-113 and IE1/IE2 was also unable to result in transient replication in the absence of UL84 (lane 4). Moreover, we have demonstrated specificity to the requirement for UL112-113 and IE2 in stimulating replication in cooperation with UL84, UL69, and the core components, since UL36-38 was unable to individually substitute for either UL112-113 or IE2 (lanes 6 to 8). Recall that both UL112-113 and IE1/IE2, however, were individually dispensable in the omission assay when the remaining four auxiliary loci were present (Fig. 1 and 2). This most likely can occur only when the conditions for replication are optimal, i.e., in the presence of all of the auxiliary components plus UL69. However, when the replication conditions are suboptimal, i.e., in the absence of some auxiliary components, the minimal requirements to detect a replication event are more strict. Our interpretation is that UL84 is a key viral polypeptide essential to promote HCMV DNA synthesis, although UL112-113 and IE1/IE2 are also critical to obtain detectable replication under certain conditions.

Identification of individual HCMV auxiliary components dispensable for *ori*Lyt-dependent DNA synthesis in cotransfection assays in HF cells. Vero cells have provided an excellent system with which to monitor the replication requirements for HSV-1 (10, 93), EBV (30, 31), and now HCMV. However, the viral components necessary to mediate origin-directed DNA replication may vary depending on the type of cell in which the transient assay was performed. To determine whether the viral loci essential for replication within Vero cells are distinct from those within a primary cell line, the cotransfection replication assay was duplicated in diploid HF cells. In HF cells, the necessity for UL84 function remained constant, with an RI of 0.01 (Fig. 5, lane 4), and UL112-113 and IRS1 both proved nonessential for replication function (RIs of 0.39 and 0.93, respectively) even in the absence of UL69 in the omission assay (lanes 5 and 8, respectively). However, the replication activity in the absence of only UL112-113 was 2.5-fold lower than the wild-type activity. These results are in close agreement with the replication assay data from Vero cells. Moreover, UL69 enhanced replication 2.3-fold upon addition to the core (lane 3) and auxiliary (lane 2) components in HF cells, although its ability to substitute for IE1/IE2 and UL36-38 activity in HF cells has not yet been tested. There was a direct requirement for both UL36-38 and IE1 plus IE2 for detectable activity (lanes 6 and 7), although unlike in Vero cells, IE2 alone was not sufficient (lane 9). It is likely that these IE polypeptides are required in part because of the differentiated state of the primary cell line. Taken together, both the HF cell and Vero cell cotransfection assay data provide evidence to suggest that UL84 is the most critical auxiliary protein and that it likely fulfills an essential replication function, perhaps as an initiator of HCMV *ori*Lyt DNA replication.

Formation of intranuclear replication compartments by cotransfection assays as detected by IFA is absolutely dependent on UL84. To obtain further evidence in support of the role of UL84, we used an alternative approach to screen individual transfected cells for replication compartment formation by IFA. In our experiments, antibody against the HCMV core component UL44 (polymerase accessory protein) was used to monitor replication protein localization and compartment formation. Our initial studies began with HCMV-infected HF cells to discern the localization of UL44 at 36 and 80 hpi (Fig. 6). At 36 hpi, before onset of the viral replicative cycle, the viral polymerase accessory protein was localized into well-defined prereplicative globular structures (Fig. 6a). At times during active viral DNA synthesis (80 hpi), the prereplicative structures grew in mass to generate large intranuclear compartments typical of a herpesvirus infection (Fig. 6b). Such structures did not form upon the introduction of the viral DNA polymerase inhibitor PAA to HCMV-infected cells, although the prereplicative foci remained (Fig. 6c).

We set out to determine whether similar replication compartments are created upon cotransfection of the HCMV replication machinery. Transfection of a UL44 expression plasmid into Vero cells results in a nuclear diffuse expression pattern as detected by IFA (see Fig. 8a). Alternatively, when UL44 localization was analyzed in the presence of both the core and the auxiliary components, but in the absence of the origin DNA, typical prereplicative foci developed (Fig. 7a). These globular structures were relatively small and often few in num-

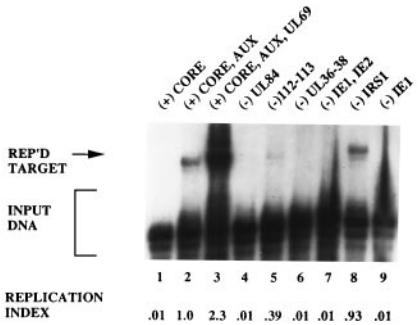

FIG. 5. HCMV *ori*Lyt DNA replication depends on UL84, UL36-38, and IE1/IE2 in an omission assay in the presence of the remaining 10 viral components in cotransfection replication assays in HF cells in the absence of UL69. HF cells were cotransfected with the core replication expression plasmids, pSP50 *ori*Lyt, and the five HCMV auxiliary (AUX) replication expression plasmids minus the one component listed above each lane. Southern blot analysis of the *Kpn*I- and *Dpn*I-digested transient replication assay was performed as described for Fig. 1. The replicated and unreplicated DNA are denoted by REP'D TARGET and INPUT DNA, respectively, and the RI values are indicated at the bottom.

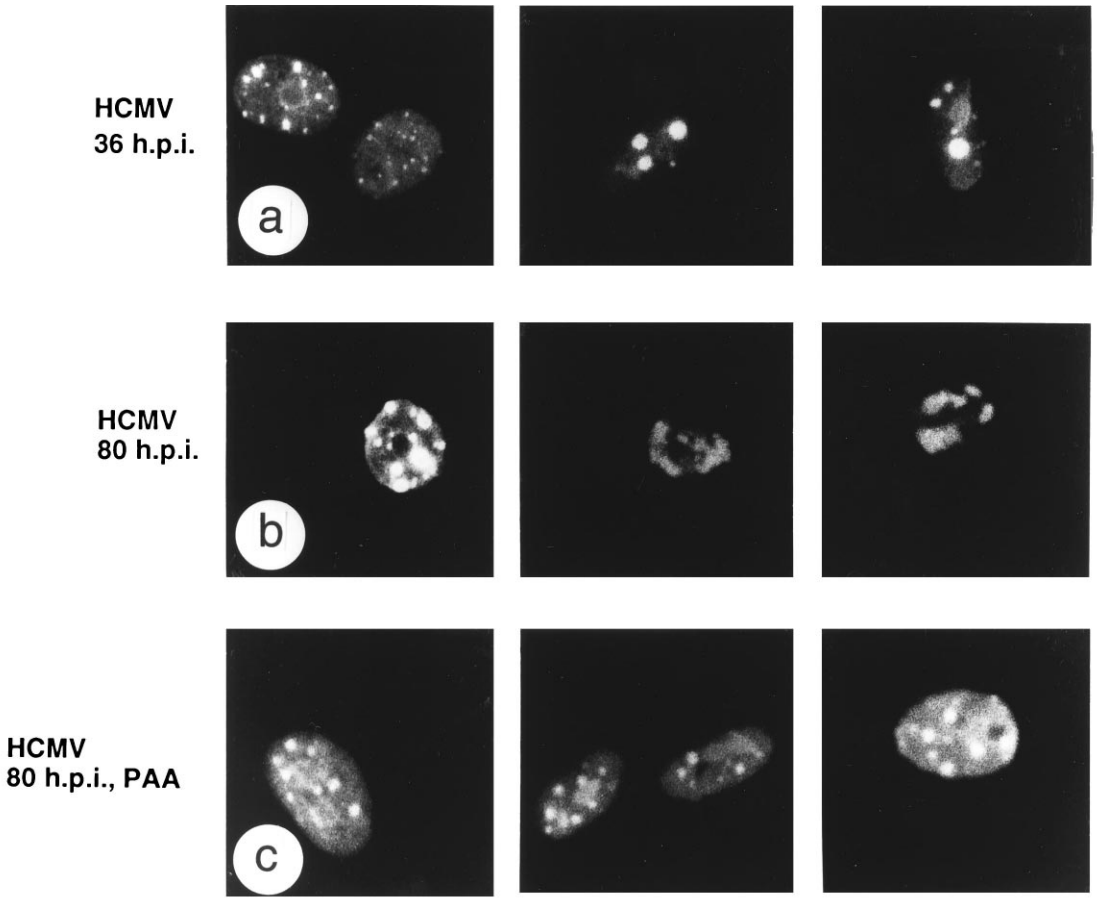

FIG. 6. Formation of both complete intranuclear replication compartments and prereplication structures detected by IFA in HCMV-infected HF Cells. HF cells were infected with HCMV(AD169) and examined for the formation of replication structure by using a fluorescein isothiocyanate-labeled mouse MAb to the HCMV polymerase accessory protein UL44. (a) HCMV-infected HF cells at 36 hpi; (b) HCMV-infected HF cells at 80 hpi; (c) HCMV-infected HF cells treated with PAA at 0 hpi and harvested at 80 hpi.

ber, similar to those formed in infection at 36 hpi (Fig. 6a). Therefore, formation of these prereplicative globular sites was not dependent on the presence of the viral origin, and they may serve as primed replisomes awaiting the loading of the origin DNA. Upon addition of the *ori*Lyt DNA to the complete HCMV replication machinery (core plus auxiliary), these prereplication sites grew in size culminating in the formation of large, amorphous, typical intranuclear compartments (Fig. 7b). Thus, in the presence of *ori*Lyt DNA, the HCMV replication machinery mediated formation of similar compartments that appeared to be indistinguishable from those formed during viral infection (Fig. 6b). Evidently, the development of complete replication compartments commenced only upon the addition of viral origin DNA. This report is the first demonstration of the observation of herpesvirus replication compartment formation in the absence of the other components provided by viral infection. Moreover, we have exploited this system to identify UL84 as a putative viral initiator of HCMV DNA replication.

The addition of the viral DNA polymerase inhibitor PAA either to HCMV-infected cells (Fig. 6c) or to Vero cells transfected with the full set of replication components plus origin DNA (Fig. 7c) effectively disrupted the formation of the replication compartments in both cases and apparently arrested the process at an early prereplication formation stage, as judged by the limited size of redistributed globular structures. Importantly, when UL84 was omitted from the complete set of replication components, including *ori*Lyt (Fig. 7d), the ability to recompartmentalize into replication compartments was abrogated, leading to arrest at the level of small prereplicative globular structures. These structures are both smaller and less numerous than the typical prereplication foci, although they are not dependent on UL84 for their formation. Additionally, in control experiments, UL44 failed to organize or redistribute into nuclear structures when in the presence of UL84 alone (Fig. 7e) or in the presence of the entire set of auxiliary components (Fig. 8b). However, when cotransfected with all five of the other core replication machinery genes, UL44 redistributed into numerous nuclear dots, most likely the result of protein-protein association with one or more of the core replication machinery proteins (Fig. 8c). Additionally, UL44 cotransfected with the viral origin plasmid pSP38 alone (Fig. 8d) failed to demonstrate any intranuclear organization and remained nuclear diffuse. Therefore, UL44 localization into higher-ordered structures was terminated in the absence of UL84, and both UL84 and target origin DNA were absolutely required for active replication compartment formation, providing strong evidence confirming that UL84 is a key factor involved in the initiation of HCMV *ori*Lyt DNA replication.

Additionally, we have identified the same minimal auxiliary components necessary to replicate HCMV *ori*Lyt by monitoring replication compartment formation in the IFA as deter-

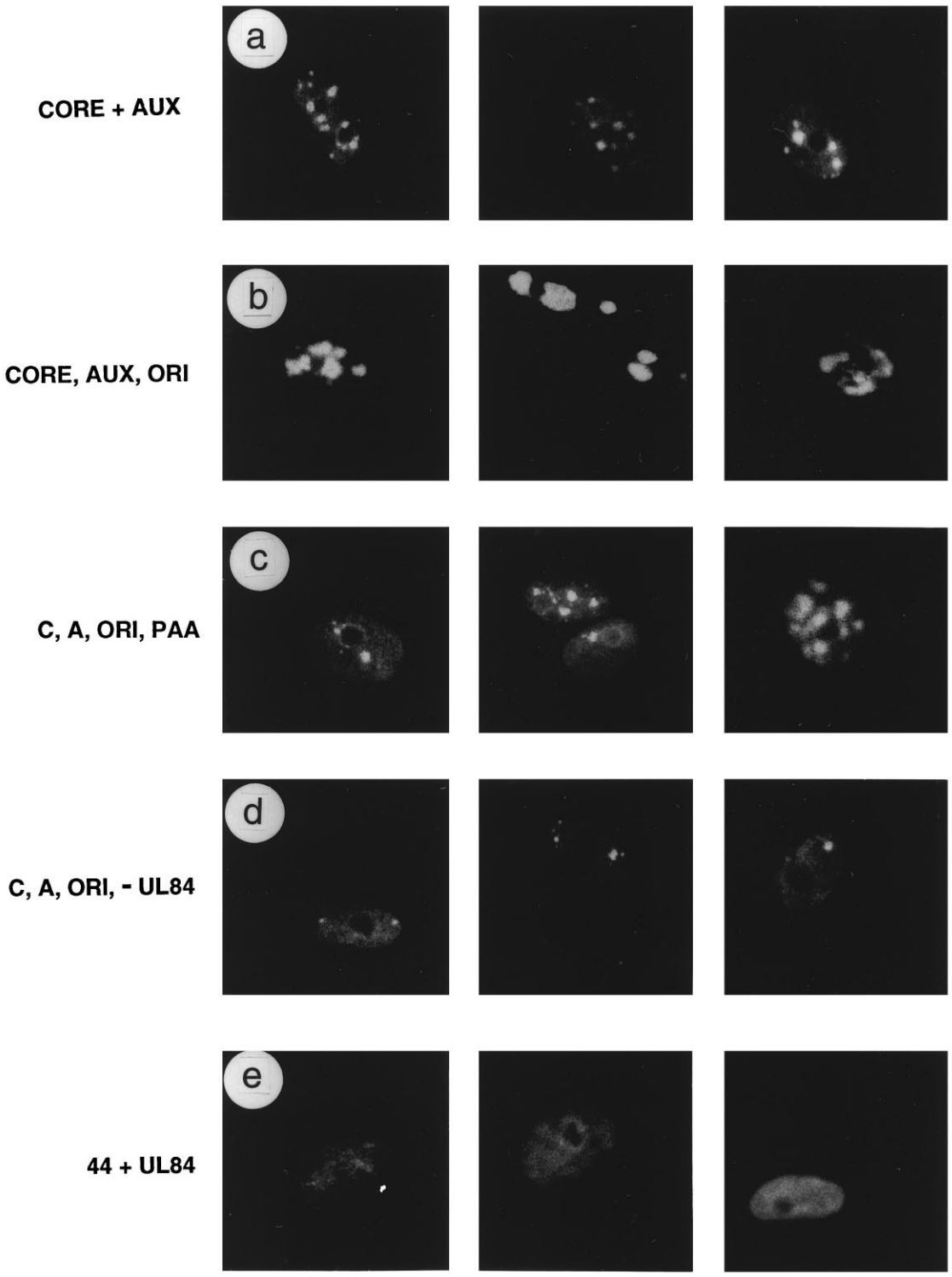

FIG. 7. Formation of replication compartments and prereplication foci detected by IFA in cotransfection assays depends on the presence of both UL84 and *ori*Lyt DNA. Vero cells were transfected with the specific replication machinery genes detailed below (minus UL69) and examined for replication structure formation by using a fluorescein isothiocyanate C-labeled mouse MAb to the HCMV polymerase accessory protein UL44. (a) Three examples of cells receiving UL44 with the complete set of both core and auxiliary (AUX) expression plasmids but without the origin plasmid (b) UL44 with both the complete set of core and auxiliary expression plasmids as well as *ori*Lyt (ORI) DNA in plasmid pSP38; (c) same as panel b except treated with PAA for the duration of the experiment; (d) same as panel b except that UL84 was omitted from the transfection; (e) UL44 with UL84 only.

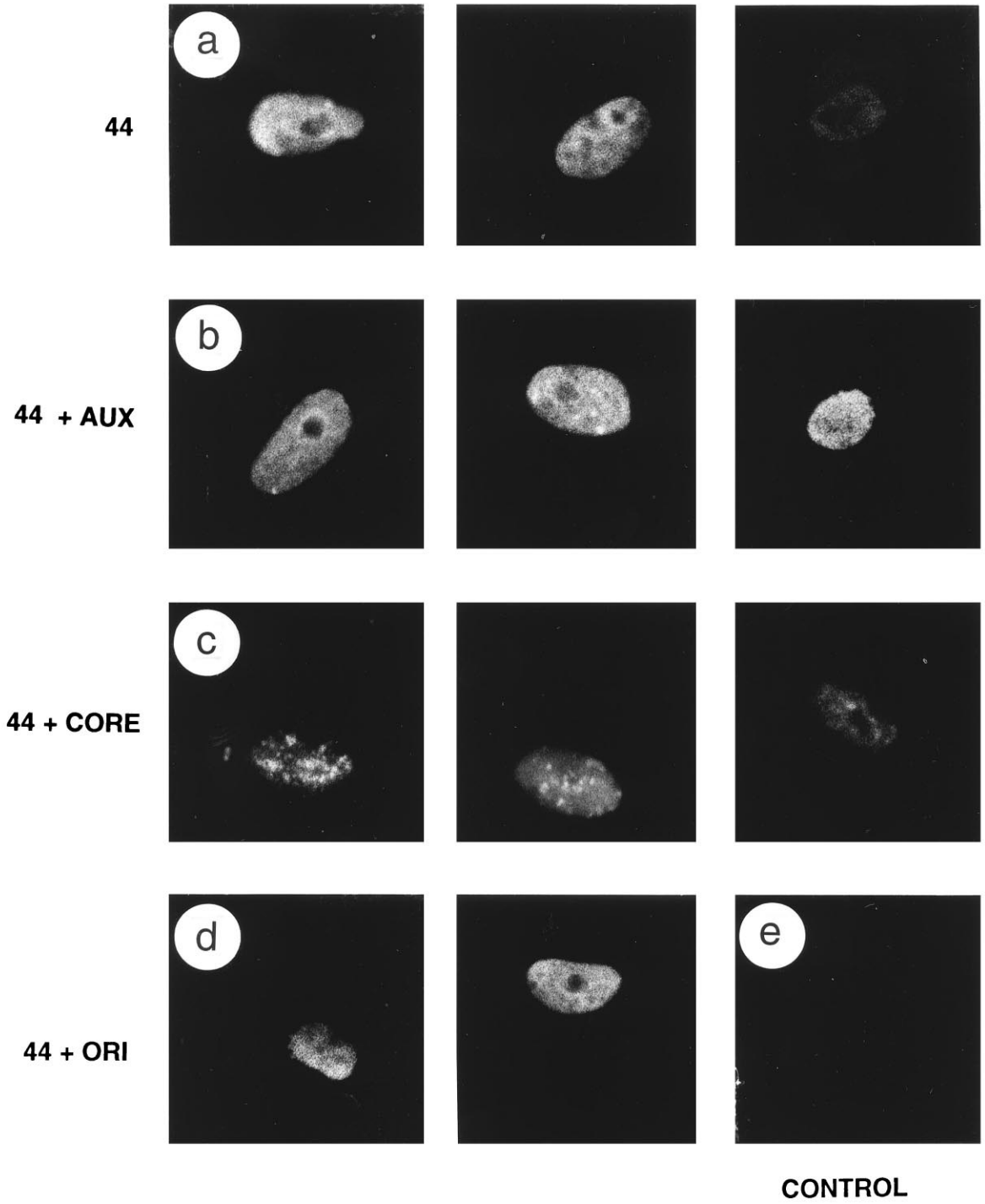

FIG. 8. Absence of intranuclear reorganization of UL44 as detected by IFA in cotransfection assays with the core replication proteins, auxiliary components, or *ori*Lyt DNA alone. Vero cells were transfected with the specific replication machinery genes detailed below and examined for replication structure formation by using a fluorescein isothiocyanate-labeled mouse MAb to the HCMV polymerase accessory protein UL44. (a) UL44 only; (b) UL44 with the five HCMV auxiliary (AUX) replication expression plasmids; (c) UL44 with the five other HCMV core replication expression plasmids; (d) UL44 with *ori*Lyt (ORI) pSP38 plasmid DNA only; (e) negative control for the anti-UL44 antibody with pSG5-transfected cells.

mined in the transient replication assay. UL84 plus UL112-113, IE2, UL69, and the core replication machinery were the minimal components necessary for the detection of wild-type replication compartment structures (data not shown). Moreover, omission of either UL112-113 or IE2 from this minimal cotransfection mixture showed a phenotype identical to that of removing UL84; this result indicates a codependence on these three auxiliary loci, in the absence of IRS1, UL36-38, and IE1, for the initiation and amplification of HCMV *ori*Lyt. These findings are in agreement with the transient replication assay

results shown in Fig. 4, which determined the minimal auxiliary replication proteins required to amplify the HCMV *ori*Lyt.

Cotransfection-generated HCMV replication compartments actively synthesize DNA and incorporate BUdR. In an attempt to demonstrate that the replication compartments visualized in individual cells cotransfected with HCMV core, auxiliary, and origin components delineate sites of active DNA synthesis, a BUdR pulse was used before IFA analysis. BUdR, a thymidine analog, can be incorporated specifically into DNA in place of thymidine. In untransfected Vero cells, approximately 25% incorporate BUdR in a pulse and can be identified with an anti-BUdR antibody, representing those cells in the S phase of the cell cycle that are actively synthesizing cellular DNA. The speckled BUdR incorporation pattern representing cellular DNA synthesis can easily be distinguished from the large aggregates indicative of viral DNA replication structures. Although some cellular DNA replication may occur within these virus-induced intranuclear compartments, such structures typical of viral infection do not occur within uninfected cells even in S phase. Although we were limited in the ability to perform double-labeling IFA analysis of UL44 and BUdR because of the source of MAbs available, single-labeling studies afforded the opportunity to assay for active DNA synthesis within the transfection-induced replication factory structures. A slide culture of Vero cells transfected with the complete set of replication machinery including *ori*Lyt (core, auxiliary, and origin) was pulsed with BUdR for 20 min prior to splitting into two wells for IFA analysis. One well was reacted with antibody to UL44, and the other, identical well was incubated with an anti-BUdR antibody for comparison. The distribution of UL44 within reconstituted replication compartments is depicted in Fig. 9a; Fig. 9b demonstrates BUdR incorporation patterns. Note that a subset of the cells labeled with anti-BUdR (arrows in Fig. 9b) show structures similar to the replication compartments identified with the anti-UL44 antibody (Fig. 9a). In addition, some (presumably S-phase cells) were competent to incorporate the nucleotide analog into the speckled pattern typical of cellular replication (those cells in Fig. 9b not marked with an arrow). Additionally, cells transfected with vector DNA (pSG5) failed to form any of the intranuclear structures typical of active viral DNA synthesis as detected by BUdR labeling (Fig. 9c), thus reaffirming our interpretation that the UL84-dependent compartment formation represents authentic sites of viral DNA replication.

DISCUSSION

The mechanism of initiation of HCMV DNA replication is one of the least understood aspects of this pathogenic virus. A key step toward understanding such a process relies on the identification of the minimal set of viral components responsible for directing the amplification event. In this report, we have identified an absolute requirement for the presence of the UL84 gene product in transient transfection replication assays and suggest that it should be considered a strong candidate for the expected viral protein that promotes the initiation process (although that need not imply that it is a DNA-binding protein).

In Vero cells, the IRS1, IE1, IE2, and UL112-113 auxiliary genomic loci can be dispensed with individually while maintaining replication function when the core replication genes and remaining auxiliary loci are expressed under the control of heterologous constitutive transcriptional signals, thus indicating a potentially indirect involvement for these gene products in viral DNA synthesis. Additionally, we identified a strict requirement for both UL84 and UL36-38 in the *ori*Lyt-directed omission assay for DNA replication. Nevertheless, the requisite presence of UL36-38, but not UL84, could be dispensed with upon supplementation of the transient replication assay with the viral transactivator UL69. This result suggests that the UL84 gene product is the only viral auxiliary factor obligatorily required for HCMV DNA replication in the presence of all other 10 replication components (Table 3).

In infected cells, UL69 accumulates in intranuclear inclusions that most likely represent viral DNA replication compartments (88). Moreover, the distribution of UL69 to these replication centers may indicate an intimate association of UL69 with the replication machinery or nuclear matrix for efficient DNA replication. The transient cotransfection assay indicated that UL69 was able to compensate for low replication activities (in the absence of IE1 and IE2 or UL36-38) by rescuing replication to a level comparable to that of the wild type; however, the UL69 gene product was unable to compensate for the stimulatory contribution of UL112-113 to the replication assay. This result further supports the suggestion that the mechanism of action of UL69 may be dependent on its associated transactivation or purported posttranslational properties and, moreover, implicates the function of UL112-113 in HCMV DNA replication to be possibly other than transcriptional or posttranscriptional activation.

Furthermore, we demonstrated a functional complementation of the HCMV core replication machinery by substitution with the EBV core replication machinery in Vero cells upon the addition of the HCMV auxiliary expression plasmids. In fact, the heterologous EBV core components were able to directly mediate replication of the HCMV *ori*Lyt simply upon the addition of the UL84 gene product alone. The UL36-38 gene products were unable to functionally substitute for UL84 to produce a similar activity, therefore implicating only UL84 as absolutely necessary for *ori*Lyt-dependent DNA synthesis in the transient replication assay and suggesting a potential role in promoting the initiation of HCMV DNA replication. Ironically, the constitutively expressed EBV core replication machinery was more adept at facilitating this replication event than was the HCMV core replication machinery itself, since UL84 alone could not mediate *ori*Lyt DNA replication when cotransfected with the HCMV core machinery. This could perhaps be explained in part by differences in core replication protein expression, mRNA stability, a more intimate association of EBV components with the cellular DNA synthesis machinery, or a greater requirement for UL112-113, for example, with the HCMV core components.

A precise definition of an HCMV protein(s) that promotes the initiation of DNA replication may not have been possible in assays using HF cells because UL69 may be unable to directly compensate for the absence of the combined activities of UL36-38 and IE1/IE2 in these cells compared with Vero cells, thus limiting the potential usefulness of HF cells in this regard. Also, the use of Vero cells offered the opportunity to cotransfect the 11 expression plasmids along with target origin DNA in a more efficient manner than in HF cells and therefore allowed us to focus on the requirements of replication per se, rather than on factors which contribute only to efficient gene expression. The UL36-38 and IE1/IE2 genomic loci, in addition to UL84, were all found to be essential auxiliary proteins for transient *ori*Lyt replication in the omission assay in the primary cell line (Table 3). Additionally, the removal of UL112-113 from the replication assay severely reduced the detectable level of *ori*Lyt amplification. Recently, Iskenderian et al. (42) argued that the cooperative interactions observed between some of the auxiliary factors are critical to either produce a synergistic enhancement of HCMV early promoters

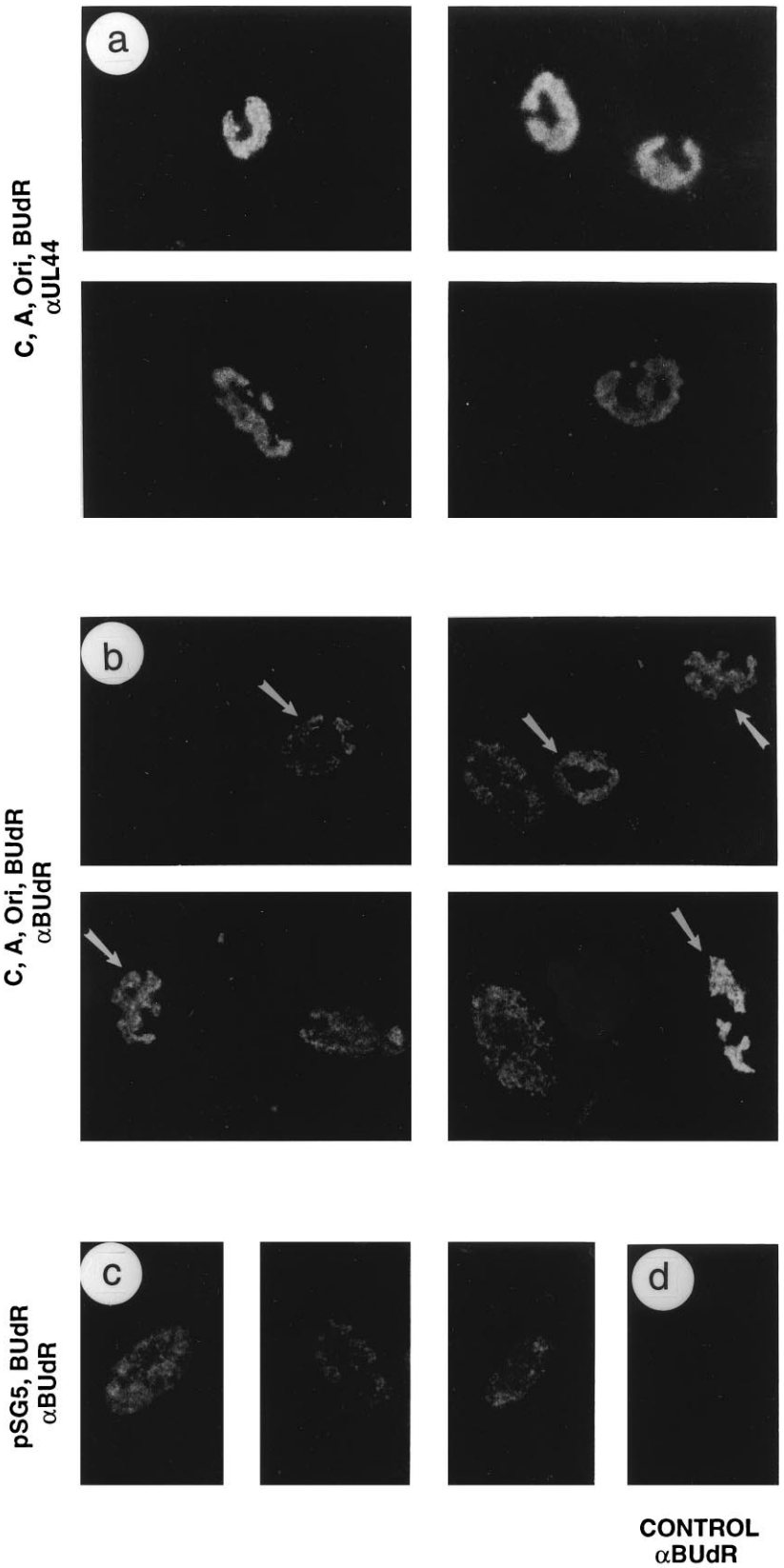

FIG. 9. Visualization of active DNA synthesis within cotransfection-induced viral replication structures by BUdR residue incorporation. Vero cells transfected with the complete set of replication expression plasmids plus *ori*Lyt (Ori) were pulse-labeled for 30 min with BUdR prior to IFA. Individual cells were screened for replication compartment formation with an anti-UL44 MAb (αUL44) and for active DNA synthesis within the viral replication-related intranuclear structures with an anti-BUdR MAb (αBUdR). (a) Anti-UL44 antibody to identify intranuclear compartments containing viral replication proteins; (b) anti-BUdR antibody to identify sites of ongoing DNA synthesis (arrows indicate those structures which resemble viral replication compartments); (c) anti-BUdR antibody showing typical speckled BUdR incorporation patterns found in 25% of untransfected cells; (d) negative control for the anti-BUdR antibody with pSG5-transfected cells.

TABLE 3. Cell-specific differences in requirements for HCMV auxiliary proteins in the presence of the six core replication genes

Viral gene	Requirement for *ori*Lyt replication[a]				
	Vero cells				HF cells
	HCMV core		EBV core, −UL69 (Fig. 3)	HCMV core +UL69[c] (Fig. 4)	HCMV core −UL69[b] (Fig. 5)
	−UL69[b] (Fig. 1)	+UL69[b] (Fig. 2)			
UL36-38	+	−	−	−	+
IRS1	−	−	−	−	−
IE1/IE2 (UL122/123)	−[d]	−	−	+[e]	+
UL112-113	−[d]	−[d]	−	+	−[d]
UL84	+	+	+	+	+

[a] +, the viral locus is absolutely required for HCMV *ori*Lyt DNA replication; −, the viral locus is not directly required for HCMV *ori*Lyt DNA replication.
[b] Data are from the omission assay in the presence of all auxiliary components minus one factor.
[c] Data are from the minimal replication assay experiment.
[d] Significantly enhances replication activity but was not absolutely essential.
[e] IE2 was essential in the minimal replication assay.

or create a local environment suitable for efficient synthesis of viral DNA replication proteins in the naturally permissive HF cells. Note that they also failed to associate any transactivation function with UL84 expression, consistent with our interpretation that its role in replication is potentially direct (42). Thus, the requirement for such transactivators in DNA replication can most likely be explained by a dependence on cooperative regulatory interactions of multiple loci for the efficient expression of cellular and viral replication proteins.

The cooperative interplay between IE polypeptides like UL36-38 and IE1/IE2, which can activate expression of certain cellular as well as viral replication genes, may likely be crucial to allow viral replication to occur in differentiated cell types. To initiate permissive infection, HCMV generally depends greatly on the host cell machinery to transcribe and translate the IE gene products, which in turn have demonstrated the ability to alter the expression of cellular genes. For example, stimulation of *hsp70* mRNA synthesis occurs within a few hours after infection and is consistent with transactivation by IE gene products (66). Use of Vero cells in our analysis allowed the identification of an absolute dependence on only a single auxiliary component, UL84, for viral DNA synthesis, provided that UL69 plus all other auxiliary components and the core replication machinery were also present. Likewise, the requirement for different viral components to mediate DNA replication in in vivo situations (e.g., monocytes, macrophages, and endothelial cells) may be unique to each cell lineage.

The importance of associated transcription factors to the process of DNA replication can be underscored by the results of studies in numerous other viral systems, including the demonstration of direct effects by cellular NF1 for adenovirus (13), E1 and E2 for bovine papillomavirus (48), Zta, Mta, and Rta for EBV (31, 48, 68, 69), and large T antigen for SV40 (26, 35). The contributions of multiple viral transcriptional activators obviously plays a key but still indirect role in mediating the process of HCMV DNA replication as is demonstrated in transient transfection-replication assays. Initially, we identified an absolute dependence (in Vero cells) upon both UL36-38 and UL84 for function in the omission assay. The UL36-38 region is unlikely to contribute a direct effect to the process of DNA replication because when the efficiency of gene expression in the system is optimal (i.e., in the presence of UL69), this requirement was abolished. Moreover, it was not directly required for replication in the minimal auxiliary component assay in the presence of UL69 or with the EBV core components. Perhaps, in addition to its viral gene transactivator role, UL36-38 (and possibly UL69) helps to recruit the viral replication machinery into the active region of the origin, mediates interactions with UL84 and the replication proteins, or is responsible for transactivating cellular replication genes critical for HCMV DNA synthesis. Similar functional roles may be likely for UL112-113 and IE1/IE2 in enhancing *ori*Lyt DNA replication to a level detectable by Southern blot analysis, as evidenced by their requirement in the minimal replication assay. Additionally, these auxiliary proteins may function in a more direct replication capacity rather than simply as transactivators of gene expression, although Iskenderian et al. (42) demonstrated that cooperation between the entire set of auxiliary components can increase expression from the SV40 early promoter 250% over that of the major IE proteins alone (42). Such increases in gene expression could significantly impact the replication efficiency as we have demonstrated.

We also determined the minimal auxiliary protein components essential to direct amplification of the HCMV *ori*Lyt in a transient assay in Vero cells. Three auxiliary factors were identified as essential in this experiment: UL84, UL112-113, and IE2. Thus, replication of HCMV is significantly different from that of HSV-1 and requires additional auxiliary components to produce detectable levels of replication. Overall, DNA replication appears to be more complex in HCMV than in other viral systems and may potentially employ more than one viral initiator polypeptide and/or may likely utilize cellular factors to facilitate this process. The direct involvement of cellular factors in the initiation of replication is feasible since HCMV, unlike HSV-1, stimulates cellular DNA and RNA synthesis (4, 5, 15, 27, 43, 66, 77, 94), and moreover, HCMV *ori*Lyt possesses numerous redundant sequence-specific motifs similar in organization to cellular replicons (1, 25). We suggest that when the conditions for replication are limiting, or suboptimal, additional auxiliary loci are necessary to detect a replication event at the level of Southern blot analysis. We speculate that although the UL112-113 phosphoproteins may indeed be responsible for activating key cellular replication fork proteins, it is equally likely that this family of proteins is directly involved in the formation and stabilization of the replication complex. Experiments are in progress to define the obligatory function of UL112-113 in the minimal replication assay.

The lack of a well-characterized nuclear localization signal within the UL84 amino acid sequence may delineate the requirement for IE2 in the transient replication assay. Spector and Tevethia (72) have elegantly demonstrated that UL84 and IE2 exist in a complexed form in infected cells. They speculated that one function of this interaction may be for IE2,

which possesses two nuclear localization signals (58), to serve as a chaperone polypeptide to target UL84 to the nucleus, the active site of DNA replication. Perhaps IE2 is responsible for targeting a substantial portion of UL84 to the replication fork for the initiation of DNA synthesis but it is not required directly for replication activity itself. Experiments are under way to test this hypothesis.

We have also observed here that apparently complete replication compartments detectable by IFA can be formed in transiently transfected cells upon the addition of the full set of replication machinery components and the viral origin DNA. Unexpectedly, these structures very closely resemble those identified at late times (80 hpi) in HCMV-infected cells. In addition, in the absence of *ori*Lyt DNA, the core and auxiliary components alone generated prereplicative structures typical of HCMV-infected cells at 36 hpi. The large intranuclear compartments reconstituted by cotransfection appear likely to represent authentic sites of ongoing viral DNA replication, as determined by their disruption in the presence of PAA and by BUdR-labeling studies. Additionally, the cotransfection generated replication compartment structures retained an absolute requirement for UL84 and *ori*Lyt, as did the in vitro replication assays. Formation of such structures was ablated upon the omission of UL84, leading to formation of only the smaller prereplication foci, which are similar to those demonstrated for HSV infection in the presence of DNA synthesis inhibitors (19).

Overall, the results of all four of our experimental assay approaches each imply an essential function for UL84 in the initiation process of HCMV DNA synthesis. UL84 may play a direct role in the formation of the replication fork initiation complex, although it need not serve as a dedicated origin-binding protein. Alternatively, it may be critical for the expression and/or recruitment of cellular initiator polypeptides into a replisome complex, or it may be responsible for promoting the initiation of HCMV DNA replication by a novel method unfamiliar to most viral systems. Additionally, our data suggest that UL112-113 and IE2, although not essential for replication activity in the omission assay, may collectively serve to stimulate the generation or possibly stabilization of this initiation complex.

ACKNOWLEDGMENTS

We thank D. Anders, G. Pari, and A. Colberg-Poley for plasmids; D. J. Spector for HCMV(AD169); R. Schnaar for help with densitometry; D. Ciufo for technical advice and helpful discussions; and S. Heaggens for assistance in manuscript preparation.

This work was supported by Public Health Service grant R01 AI31454 from the National Institutes of Health (G.S.H.) and a postdoctoral fellow stipend from Anticancer Drug Development training grant T32 CA09243 (R.T.S.).

REFERENCES

1. **Anders, D. G., M. A. Kacica, G. 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.
2. **Anders, D. G., and S. M. Punturieri.** 1991. Multicomponent origin of cytomegalovirus lytic-phase DNA replication. J. Virol. **65:**931–937.
3. **Baldanti, F., M. R. Underwood, S. C. Stanat, K. K. Biron, S. Chou, A. Sarasini, E. Silini, and G. Gerna.** 1996. Single amino acid changes in the DNA polymerase confer foscarnet resistance and slow-growth phenotype, while mutations in the UL97-encoded phosphotransferase confer ganciclovir resistance in three double-resistant human cytomegalovirus strains recovered from patients with AIDS. J. Virol. **70:**1390–1395.
4. **Boldogh, I., S. AbuBakar, and T. Albrecht.** 1990. Activation of proto-oncogenes: an immediate early event in human cytomegalovirus infection. Science **247:**561–564.
5. **Boldogh, I., S. AbuBakar, C. Z. Deng, and T. Albrecht.** 1991. Transcriptional activation of cellular oncogenes *fos*, *jun*, and *myc* by human cytomegalovirus. J. Virol. **65:**1568–1571.
6. **Borowiec, J. A., F. B. Dean, P. A. Bullock, and J. Hurwitz.** 1990. Binding and unwinding—how T antigen engages the SV40 origin of DNA replication. Cell **60:**181–184.
7. **Bruckner, R. C., J. J. Crute, M. S. Dodson, and I. R. Lehman.** 1991. The herpes simplex virus 1 origin binding protein: a DNA helicase. J. Biol. Chem. **266:**2669–2674.
8. **Buisson, M., E. Manet, M. C. Trescol-Biemont, H. Gruffat, and A. Sergeant.** 1989. The Epstein-Barr virus (EBV) early protein EB2 is a posttranscriptional activator expressed under the control of EBV transcription factors EB1 and R. J. Virol. **63:**5276–5284.
9. **Bush, M., D. R. Yager, M. Gao, K. Weisshart, A. I. Marcy, D. M. Coen, and D. M. Knipe.** 1991. Correct intranuclear localization of herpes simplex virus DNA polymerase requires the viral ICP8 DNA-binding protein. J. Virol. **65:**1082–1089.
10. **Challberg, M.** 1986. A method for identifying the viral genes required for herpesvirus DNA replication. Proc. Natl. Acad. Sci. USA **83:**9094–9098.
11. **Challberg, M. D., and T. J. Kelly.** 1989. Animal virus DNA replication. Annu. Rev. Biochem. **58:**671–717.
12. **Chee, M. S., et al.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154:**125–169.
13. **Chen, M., N. Mermod, and M. Horwitz.** 1990. Protein-protein interactions between adenovirus DNA polymerase and nuclear factor 1 mediate formation of the DNA replication preinitiation complex. J. Biol. Chem. **265:**18634–18642.
14. **Cho, M.-S., G. Milman, and S. D. Hayward.** 1985. A second Epstein-Barr virus early antigen gene in *Bam*HI fragment M encodes a 48- to 50-kilodalton nuclear protein. J. Virol. **56:**860–866.
15. **Colberg-Poley, A. M., and L. D. Santomenna.** 1988. Selective induction of chromosomal gene expression by human cytomegalovirus. Virology **166:**217–228.
16. **Colberg-Poley, A. M., L. D. Santomenna, 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.
17. **Dean, F. B., P. Bullock, Y. Murakami, C. Wobbe, L. Weissback, and J. Hurwitz.** 1987. SV40 DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. Proc. Natl. Acad. Sci. USA **84:**16–20.
18. **Deb, S., A. DeLucia, A. Koff, S. Tsui, and P. Tegtmeyer.** 1986. The adenine-thymine domain of the simian virus 40 core origin detects DNA bending and coordinately regulates DNA replication. Mol. Cell. Biol. **6:**4578–4584.
19. **de Bruyn Kops, A., and D. M. Knipe.** 1988. Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. Cell **55:**857–868.
20. **de Bruyn Kops, A., and D. M. Knipe.** 1994. Preexisting nuclear architecture defines the intranuclear location of herpesvirus DNA replication structures. J. Virol. **68:**3512–3526.
21. **DePamphilis, M. L.** 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. Cell **52:**635–638.
22. **DePamphilis, M. L.** 1993. Origins of DNA replication in metazoan chromosomes. J. Biol. Chem. **268:**1–4.
23. **deVilliers, J., W. Schaffner, C. Tyndall, S. Lupton, and R. Kamen.** 1984. Polyoma virus DNA replication requires an enhancer. Nature (London) **312:**242–246.
24. **Diffley, J. F., and B. Stillman.** 1990. The initiation of chromosomal DNA replication in eukaryotes. Trends Genet. **6:**427–432.
25. **Dobbs, D. L., W. L. Shaiu, and R. M. Benbow.** 1994. Modular sequence elements associated with origin regions in eukaryotic chromosomal DNA. Nucleic Acids Res. **22:**2479–2489.
26. **Dornreiter, I., L. F. Erdile, I. U. Gilbert, D. V. Winkler, T. J. Kelly, and E. Fanning.** 1992. Interaction of DNA polymerase alpha-primase with cellular replication protein A and SV40 T antigen. EMBO J. **11:**769–776.
27. **Dudding, L., S. Haskill, B. D. Clark, P. E. Auron, S. Sporn, and E. S. Huang.** 1989. Cytomegalovirus infection stimulates expression of monocyte-associated mediator genes. J. Immunol. **143:**3343–3352.
28. **Elias, P., E. O'Donnell, E. S. Mocarski, and I. R. Lehman.** 1986. A DNA binding protein specific for the origin of replication of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **83:**6322–6326.
29. **Fiala, M., J. E. Payne, and T. V. Berne.** 1975. Epidemiology of cytomegalovirus infection after transplantation and immunosuppression. J. Infect. Dis. **132:**421–433.
30. **Fixman, E. D., G. S. Hayward, and S. D. Hayward.** 1992. *trans*-acting requirements for replication of EBV ori-lyt. J. Virol. **66:**5030–5039.
31. **Fixman, E. D., G. S. Hayward, and S. D. Hayward.** 1995. Replication of Epstein-Barr virus oriLyt: lack of a dedicated virally encoded origin-binding protein and dependence on Zta in cotransfection assays. J. Virol. **69:**2998–3006.
32. **Gavin, K. A., M. Hidaka, and B. Stillman.** 1996. Conserved initiator proteins in eukaryotes. Science **270:**1667–1671.
33. **Glenn, J.** 1981. Cytomegalovirus infection following renal transplantation. Rev. Infect. Dis. **3:**1151–1178.
34. **Goodrich, L. D., P. A. Schaffer, D. I. Dorsky, C. S. Crumpacker, and D. S.**

Parris. 1990. Localization of the herpes simplex virus type 1 65-kilodalton DNA-binding protein and DNA polymerase in the presence and absence of viral DNA synthesis. J. Virol. **64:**5738–5749.
35. **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.
36. **Guo, Z. S., C. Gutierrez, U. Heine, J. M. Sogo, and M. L. DePamphilis.** 1989. Origin auxiliary sequences can facilitate initiation of simian virus 40 DNA replication in vitro as they do in vivo. Mol. Cell. Biol. **10:**1719–1728.
37. **Hamzeh, F. M., P. S. Leitman, 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.
38. **He, Y. S., L. Xu, and E.-S. Huang.** 1992. Characterization of human cytomegalovirus UL84 early gene and identification of its putative protein product. J. Virol. **66:**1098–1108.
39. **Heilbronn, R., and H. zur Hausen.** 1989. A subset of herpes simplex virus replication genes induces DNA amplification within the host cell genome. J. Virol. **63:**3683–3692.
40. **Ho, M.** 1982. Human cytomegalovirus infections in immunosuppressed patients. Curr. Top. Infect. Dis. **1982:**171–204.
41. **Huang, R.-Y., and D. Kowalski.** 1993. A DNA unwinding element and an ARS consensus comprise a replication origin within a yeast chromosome. EMBO J. **12:**4521–4531.
42. **Iskenderian, A. C., L. Huang, A. Reilly, R. M. Stenberg, and D. G. Anders.** 1996. Four of eleven loci required for transient complementation of human cytomegalovirus DNA replication cooperate to activate expression of replication genes. J. Virol. **70:**383–392.
43. **Jault, F. M., J. M. Jault, F. Ruchti, E. A. Fortunato, C. Clark, J. Corbeil, D. D. Richman, and D. H. Spector.** 1995. Cytomegalovirus infection induces high levels of cyclins, phosphorylated Rb, and p53, leading to cell cycle arrest. J. Virol. **69:**6697–6704.
44. **Kenney, S., J. Kamine, E. Holley-Guthrie, E. C. Mar, J. C. Lin, D. Markovitz, and J. Pagano.** 1989. The Epstein-Barr virus immediate-early gene product, BMLF1, acts in *trans* by a posttranscriptional mechanism which is reporter gene dependent. J. Virol. **63:**3870–3877.
45. **Kouzarides, T., A. T. Bankier, S. C. Satchwell, E. Preddy, and B. G. Barrell.** 1988. An immediate early gene of human cytomegalovirus encodes a potential membrane glycoprotein. Virology **165:**151–164.
46. **Kowalski, D., and M. J. Eddy.** 1989. The DNA unwinding element: a novel, cis-acting component that facilitates opening of the *Escherichia coli* replication origin. EMBO J. **8:**4335–4344.
47. **LaFemina, R. L., and G. S. Hayward.** 1983. Replicative forms of human cytomegalovirus DNA with joined termini are found in permissively infected human cells but not in non-permissive Balb/c-3T3 mouse cells. J. Gen. Virol. **64:**373–389.
48. **Li, R., and M. R. Botchan.** 1994. Acidic transcription factors alleviate nucleosome-mediated repression of DNA replication of bovine papillomavirus type 1. Proc. Natl. Acad. Sci. USA **91:**7051–7055.
49. **Liberman, P. M., P. O'Hare, G. S. Hayward, and S. D. Hayward.** 1986. Promiscuous *trans* activation of gene expression by an Epstein-Barr virus-encoded early nuclear protein. J. Virol. **60:**140–148.
50. **Liptak, L. M., S. L. Uprichard, and D. M. Knipe.** 1996. Functional order of assembly of herpes simplex virus DNA replication proteins into prereplicative site structures. J. Virol. **70:**1759–1767.
51. **Masse, M. J. O., S. Karlin, G. A. Schachtel, and E. S. Mocarski.** 1992. Human cytomegalovirus origin of DNA replication (oriLyt) resides within a highly complex repetitive region. Proc. Natl. Acad. Sci. USA **89:**5246–5250.
52. **McVoy, M. A., and S. P. Adler.** 1994. Human cytomegalovirus DNA replicates after early circularization by concatemer formation, and inversion occurs within the concatemer. J. Virol. **68:**1040–1051.
53. **Mohr, I. J., R. Clark, S. Sun, E. J. Androphy, P. MacPherson, and M. R. Botchan.** 1991. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. Science **250:**1694–1699.
54. **Nakamaru, H., T. Morita, and C. Sato.** 1986. Structural organization of replicon domains during DNA synthetic phase in the mammalian nucleus. Exp. Cell Res. **165:**291–297.
55. **Olivo, P. D., N. J. Nelson, and M. D. Challberg.** 1988. Herpes simplex virus DNA replication: the UL9 gene encodes an origin-binding protein. Proc. Natl. Acad. Sci. USA **85:**5414–5418.
56. **Pari, G. S., and D. G. Anders.** 1993. Eleven loci encoding *trans*-acting factors are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA replication. J. Virol. **67:**6979–6988.
57. **Pari, G. S., A. K. Field, and J. A. Smith.** 1995. Potent antiviral activity of an antisense oligonucleotide complementary to the intron-exon boundary of human cytomegalovirus genes UL36 and UL37. Antimicrob. Agents Chemother. **39:**1157–1161.
58. **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.
59. **Pizzorno, M. C., P. O'Hare, L. Sha, R. L. LaFemina, and G. S. Hayward.** 1988. *trans*-activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. J. Virol. **62:**1167–1179.
60. **Quinlan, M., L. B. Chen, and D. M. Knipe.** 1984. The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell **36:**857–868.
61. **Rao, H., Y. Marahrens, and B. Stillman.** 1994. Functional conservation of multiple elements in yeast chromosomal replicators. Mol. Cell. Biol. **14:**7643–7651.
62. **Roizman, B.** 1990. Herpes simplex viruses and their replication, p. 1795–1841. *In* B. N. Fields and D. M. Knipe et al. (ed.), Virology. Raven Press, New York.
63. **Rowley, A., J. H. Cocker, J. Harwood, and J. F. Diffley.** 1995. Initiation complex assembly at budding yeast replication origins begins with the recognition of a bipartite sequence by limiting amounts of the initiator, ORC. EMBO J. **14:**2631–2641.
64. **Sacks, W. R., C. C. Greene, D. P. Aschman, and P. A. Schaffer.** 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J. Virol. **55:**796–805.
65. **Samaniego, L. A., M. J. Tevethia, and D. J. Spector.** 1994. The human cytomegalovirus 86-kilodalton immediate-early 2 protein: synthesis as a precursor polypeptide and interaction with a 75-kilodalton protein of probable viral origin. J. Virol. **68:**720–729.
66. **Santomenna, L. D., and A. M. Colberg-Poley.** 1990. Induction of cellular *hsp70* expression by human cytomegalovirus. J. Virol. **64:**2033–2040.
67. **Sarisky, R. T., Z. Gao, P. M. Liberman, E. D. Fixman, G. S. Hayward, and S. D. Harward.** Evidence for a replication function associated with the activation domain of the Epstein-Barr virus Zta transactivator. J. Virol., in press.
68. **Schepers, A., D. Pich, and W. Hammerschmidt.** 1993. A transcription factor with homology to the AP-1 family links RNA transcription and DNA replication in the lytic cycle of EBV. EMBO J. **12:**3921–3929.
69. **Schepers, A., D. Pich, J. Mankertz, and W. Hammerschmidt.** 1993. *cis*-acting elements in the lytic origin of DNA replication of Epstein-Barr virus. J. Virol. **67:**4237–4245.
70. **Smith, J. A., and G. S. Pari.** 1995. Expression of human cytomegalovirus UL36 and UL37 genes is required for DNA replication. J. Virol. **69:**1925–1931.
71. **Spaete, R. R., and E. S. Mocarski.** 1985. Regulation of cytomegalovirus gene expression: α and B promoters are *trans* activated by viral functions in permissive human fibroblasts. J. Virol. **56:**135–143.
72. **Spector, D. J., and M. J. Tevethia.** 1995. Protein-protein interactions between human cytomegalovirus IE2-580aa and pUL84 in lytically infected cells. J. Virol. **68:**7549–7553.
73. **Speir, E., R. Modali, E. S. Huang, M. B. Leon, F. Shawl, T. Finkel, and S. E. Epstein.** 1994. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science **265:**391–394.
74. **Stagno, S., R. F. Pass, M. E. Dworsky, R. E. Henderson, E. G. Moore, P. D. Walton, and C. A. Alford.** 1982. Congenital cytomegalovirus infection: the relative importance of primary and recurrent maternal infection. N. Engl. J. Med. **306:**945–959.
75. **Stasiak, P. C., and E. S. Mocarski.** 1992. Transactivation of the cytomegalovirus ICP36 gene promoter requires the alpha gene product TRS1 in addition to IE1 and IE2. J. Virol. **66:**1050–1058.
76. **Stillman, B.** 1989. Initiation of eukaryotic DNA replication in vitro. Annu. Rev. Cell Biol. **5:**197–245.
77. **St. Jeor, S. C., T. B. Albrecht, F. D. Funk, and F. Rapp.** 1974. Stimulation of cellular DNA synthesis by human cytomegalovirus. J. Virol. **13:**353–362.
78. **Stinski, M. F.** 1990. Cytomegalovirus and its replication, p. 1959–2010. *In* B. N. Fields and D. M. Knipe et al. (ed.), Virology. Raven Press, New York.
79. **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.
80. **Tevethia, M. J., D. J. Spector, K. M. Leisure, and M. F. Stinski.** 1987. Participation of two human cytomegalovirus immediate early gene regions in transcriptional activation of adenovirus promoters. Virology **161:**276–285.
81. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen-related protein. Cell **13:**165–179.
82. **Tsurimoto, T., M. P. Fairman, and B. Stillman.** 1989. Simian virus 40 DNA replication in vitro: identification of multiple stages of initiation. Mol. Cell. Biol. **9:**3839–3849.
83. **Uprichard, S. L., and D. M. Knipe.** 1996. Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes. J. Virol. **70:**1969–1980.
84. **Waga, S., and B. Stillman.** 1994. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication in vitro. Nature (London) **369:**207–212.
85. **Weller, S. K.** 1991. Genetic analysis of HSV-1 genes required for genome replication, p. 105–136. *In* E. K. Wagner (ed.), Herpesvirus transcription and

its regulation. CRC Press, Boca Raton, Fla.
86. **Weston, K.** 1988. An enhancer element in the short unique region of human cytomegalovirus regulates the production of a group of abundant immediate early transcripts. Virology **162:**406–416.
87. **Wilcock, D., and D. P. Lane.** 1991. Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. Nature (London) **349:**429–431.
88. **Winkler, M., S. A. Rice, and T. Stamminger.** 1994. UL69 of human cytomegalovirus, an open reading frame with homology to ICP27 of herpes simplex virus, encodes a transactivator of gene expression. J. Virol. **68:**3943–3954.
89. **Wold, M. S., J. J. Li, and T. J. Kelly.** 1987. Initiation of simian virus 40 DNA replication in vitro: large-tumor-antigen and origin-dependent unwinding of the template. Proc. Natl. Acad. Sci. USA **84:**3643–3647.
90. **Wong, S., and P. Schaffer.** 1991. Elements in the transcriptional regulatory region flanking herpes simplex virus type 1 *oriS* stimulate origin function. J. Virol. **65:**2601–2611.
91. **Wright, D. A., and D. H. Spector.** 1989. Posttranscriptional regulation of a class of human cytomegalovirus phosphoproteins encoded by an early transcription unit. J. Virol. **63:**3117–3127.
92. **Wright, D. A., S. I. Staprans, and D. H. Spector.** 1988. Four phosphoproteins with common amino termini are encoded by human cytomegalovirus AD169. J. Virol. **62:**331–340.
93. **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 DNA synthesis. J. Virol. **62:**435–443.
94. **Zhu, H., Y. Shen, and T. Shenk.** 1995. Human cytomegalovirus IE1 and IE2 proteins block apoptosis. J. Virol. **69:**7960–7970.