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Characterization of Nuclear Structures in Cells Infected with Herpes Simplex Virus Type 1 in the Absence of Viral DNA Replication

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Herpes simplex virus type 1 DNA replication occurs in nuclear domains termed replication compartments, which are areas of viral single-stranded DNA-binding protein (UL29) localization (M. P. Quinlan, L. B. Chen, and D. M. Knipe, Cell 36:857–868, 1984). In the presence of herpesvirus-specific polymerase inhibitors, UL29 localizes to punctate nuclear foci called prereplicative sites. Using versions of the helicase-primase complex proteins containing short peptide epitopes which can be detected in an immunofluorescence assay, we have found that the helicase-primase complex localizes to prereplicative sites and replication compartments. To determine if prereplicative site formation is dependent upon these and other essential viral replication proteins, we have studied UL29 localization in cells infected with replication-defective viruses. Cells infected with viruses that fail to express one of the three helicase-primase subunits or the origin-binding protein show a diffuse nuclear staining for UL29. However, in the presence of polymerase inhibitors, mutant-infected cells contain UL29 in prereplicative sites. Replication-defective viruses containing subtle mutations in the helicase or origin-binding proteins behaved identically to their null mutant counterparts. In contrast, cells infected with viral mutants which fail to express the polymerase protein contain prereplicative sites in the absence and presence of polymerase inhibitors. We propose that active viral polymerase prevents the formation of prereplicative sites. Models of the requirement of essential viral replication proteins in the assembly of prereplicative sites are presented.

Cellular DNA replication in eukaryotic nuclei occurs in discrete domains called replicons (26, 27). The large number of cellular gene products involved in DNA replication suggests that replicon formation may be complex. Because its genes can be manipulated, herpes simplex virus type 1 (HSV-1) may be a useful model for studying the nuclear organization of a DNA replication apparatus. Genetic analysis has resulted in the identification of replication-defective mutants representing seven complementation groups (reviewed in reference 33). These seven viral proteins are required for the amplification of plasmids containing an HSV-1 origin of replication in a transient cotransfection assay (4, 37). The essential HSV-1 replication proteins have been identified as a heterotrimeric helicase-primase complex (UL5, UL8, and UL52), a DNA polymerase (UL30) and its accessory subunit (UL42), an origin-binding protein (UL9), and a single-stranded DNA-binding protein (UL29) (reviewed in references 29 and 33). Many interactions between these proteins have been described. For example, UL5 (helicase), UL8, and UL52 (primase) interact to form a helicase-primase complex, and the polymerase and polymerase accessory protein interact to form a heterodimeric holoenzyme (6, 9, 18). Physical interactions between UL9 and UL29 and between UL9 and UL8 have been reported (1, 25). Finally, two lines of evidence suggest an interaction between the viral polymerase and UL29. First, the virus sensitivity to polymerase inhibitors can be altered by mutations which map within the UL29 gene (5). Second, the action of UL30 on a singly primed single-stranded DNA circle is stimulated by the addition of UL29 (28, 32).

HSV-1 DNA replication occurs in nuclear domains termed "replication compartments," initially identified on the basis of UL29 staining patterns in an immunofluorescence assay (31); these compartments may represent the viral equivalent of cellular replicons. A subset of the essential viral replication proteins have been shown to localize to replication compartments, specifically the origin-binding and polymerase complex proteins (14, 30). In contrast, UL5, UL8, and UL52 were reported to be present in a diffuse staining pattern in infected nuclei (30). However, as noted by the authors, the polyclonal antisera used may not have been sensitive enough to detect the subnuclear localization of these nonabundant proteins. We now show that the helicase-primase complex localizes to replication compartments in infected cells by using epitope-tagged versions of these proteins in an immunofluorescence assay.

When viral replication is blocked by HSV-specific polymerase inhibitors, such as phosphonoacetic acid (PAA) or acyclovir (ACG), UL29 localizes to numerous punctate structures in the nucleus termed "prereplicative sites" (7, 31). Prereplicative sites colabel with bromodeoxyuridine (BrdU) and likely represent areas of cellular DNA synthesis (7). The localization of UL30 and UL42 has been examined in infected cells treated with PAA; UL30 localizes weakly to prereplicative sites, and UL42 is found in a diffuse nuclear pattern (2, 16). We now report that the members of the helicase-primase complex colocalize with UL29 in prereplicative sites.

It has been proposed that prereplicative sites represent intermediates in the assembly of the viral replication apparatus on the basis of the observation that, early in infection, UL29 localizes to structures in the nucleus which resemble prereplicative sites (31). If prereplicative sites are subassemblies of viral replication proteins, one would expect that other essential viral replication proteins would be necessary for their formation. Indeed, UL29 plays a key role in prereplicative site formation. Cells infected with UL29 mutants fail to form prereplicative sites in the presence and absence of polymerase inhibitors (2, 8, 9). In contrast, UL30 is not necessary for prereplicative site formation; UL29 localizes to prereplicative

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TABLE 1. Replication-defective viral mutants[a]

Virus	Mutation	Protein affected
*hr*99	ICP6::*lacZ*[b] insertion at residue 182 (14589)[c]	UL5
*hr*99G102V	Residue 102-G→V (14829-T→A)	UL5
*hr*99K103A	Residue 103-K→A (14826–14827-TT→GC)	UL5
*hr*99R345K	Residue 345-R→K (14100–14101-CG→TT)	UL5
*hr*80	Δ residues 5–554 (20464–188150), ICP6::*lacZ* insertion	UL8
*hr*94	ICP6::*lacZ* insertion at residue 536 (21656)	UL9
*hr*27	Residue 309-F→Y (22336-A→T) and residue 311-E→G (22330-A→G)	UL9
HP66	Δ residues 212–974 (63441–65727), TK::*lacZ*[d] insertion	UL30
*hr*114	ICP6::*lacZ* insertion at residue 182 (109594)	UL52

[a] See Materials and Methods for references.
[b] *lacZ* gene under control of the HSV-1 ICP6 promoter.
[c] Sequence coordinate (24).
[d] *lacZ* gene under control of the HSV-1 thymidine kinase (TK) promoter.

sites in cells infected with viruses that fail to express UL30 (2). To determine whether the helicase-primase complex and UL9 are essential for prereplicative site formation, we have examined prereplicative site formation in cells infected with viruses that fail to express UL5, UL8, UL52, or UL9 or with viruses that contain subtle mutations in UL5 or UL9.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney fibroblasts (Vero cells; American Type Culture Collection) were propagated and maintained as described previously (35). The KOS strain of HSV-1 was used as the wild-type virus. The following mutant viruses were used in this study (Table 1) and have been described previously: *hr*94 and *hr*27 (21); *hr*99 (39); *hr*99G102V, *hr*99K103A, and *hr*99R345K (38); *hr*80 (3); *hr*114 (13); and HP66, which was kindly provided by Donald M. Coen (Harvard Medical School, Boston, Mass. [22]).

Reagents and antibodies. Glycerol gelatin, 1,4-diazobicyclo-[2.2.2]octane (DABCO), PAA, BrdU, and ACG were obtained from Sigma (St. Louis, Mo.). A monoclonal antibody recognizing BrdU incorporated into DNA was obtained from Becton Dickinson Immunocytometry Systems (San Jose, Calif.). The polyclonal antiserum 3-83 which recognizes UL29 was a generous gift of David Knipe (Harvard Medical School) (19). The monoclonal antibody AU1, which recognizes a peptide epitope of bovine papillomavirus L1 protein, was obtained from the Berkeley Antibody Company (BAbCo, Richmond, Calif.) (12). The monoclonal antibody EE, which recognizes a peptide epitope of polyomavirus medium T antigen, was generated from mouse ascites fluid by utilizing hybridoma cells generously provided by Gernot Walter (16). The monoclonal antibody KT3, which recognizes a peptide epitope of simian virus 40 large T antigen, was obtained from mouse ascites fluid by utilizing hybridoma cells generously provided by Gernot Walter (20). The secondary antibodies, fluorescein isothiocyanate-conjugated goat anti-mouse and anti-rabbit and Texas red-conjugated goat anti-mouse and anti-rabbit antibodies, were obtained from Cappel, Organon Teknika Corporation (Durham, N.C.).

Plasmids. In this section, restriction sites are underlined and stop or start codons are in boldface. Oligonucleotides were made on a Cyclone DNA synthesizer (Biosearch Inc., Burlington, Mass.). Sequence coordinates are provided in parentheses (24).

(i) Vectors. The vector designated p6NBam contains the ICP6 promoter and the UL8/UL9 polyadenylation signal and was constructed as follows. A *Bam*HI site was introduced at the 5′ end of the UL9 open reading frame (ORF) in p6UL9-119b, which contains the UL9 gene under control of the ICP6 promoter in pUC119 (21), by site-directed mutagenesis on single-stranded DNA (23). The mutagenic oligonucleotide, corresponding to the noncoding strand, was 5′-GA AAGG**CAT**TTCGGATCCAACAGACGCGGC-3′. Insertion of the *Bam*HI site was verified by DNA sequence analysis with Sequenase according to the supplier's instructions (U.S. Biochemical Corporation, Cleveland, Ohio). The resulting plasmid was digested with *Bam*HI to remove the N-terminal two-thirds of the UL9 gene to generate p6NBam.

(ii) UL5 constructs. p6UL5 contains the UL5 ORF under control of the inducible ICP6 promoter (39). An *Sph*I fragment from p6UL5 containing the UL5 coding region and the ICP6 promoter was inserted into pUC119 to generate two plasmids, p6UL5119a and p6UL5119b, which differ only in the orientation of the insert. To facilitate genetic manipulation of the UL5 coding region, *Bam*HI sites were introduced 5′ and 3′ of UL5 as follows. A *Bam*HI site was introduced 12 bp upstream of the UL5 start codon by PCR mutagenesis of p6UL5119b. The sequence of the forward primer, which introduces the *Bam*HI site, was 5′-AT ATATGGATCCCGTGGTGCGGTC-3′. The sequence of the reverse primer, which lies within the UL5 coding region, was 5′-TCTGGCGGACGG-3′ (positions 14194 to 14203). The PCR fragment was digested with *Bam*HI and *Sal*I (14237) and ligated into p6UL5119b that had been digested with *Bam*HI and *Sal*I. The resulting construct was designated pUC119-UL5. pUC119-UL5 was digested with *Bam*HI and *Sac*II (13277) to generate a fragment containing a 5′ *Bam*HI site and the UL5 coding sequences up to the *Sac*II site. This was ligated to the *Sac*II-to-*Bam*HI fragment of UL5 from p6UL5119a to reconstitute the entire UL5 ORF and provide a *Bam*HI site at the 3′ end of the UL5 ORF. This *Bam*HI fragment was excised and ligated into *Bam*HI-linearized pUC119, and the final plasmid was designated p6BamUL5.

(iii) UL8 constructs. The UL8 ORF was isolated on an *Eco*RI-to-*Xba*I fragment from pCM-UL8 (17) and inserted between the same two sites in pGEM-7Zf$^+$ (Promega, Madison, Wis.). The resulting construct was designated pGM7/UL8. The UL8 ORF was placed under control of the ICP6 promoter by using a synthetic double-stranded oligonucleotide to link the ICP6 promoter to the UL8 gene as follows. A *Sac*I-to-*Xho*I fragment from pDG2 (38) containing the ICP6 promoter was ligated to an *Eco*RV-to-*Sac*I fragment of UL8 (20463 to 17854) from pSG10-BD1 (34) with the oligonucleotide 5′-TCGAGCCCGAAACCC GCCGCCTCTGTTGAA**ATG**GACACCGCAGAT-3′ and its complement. This double-stranded oligonucleotide was designed to yield a cohesive *Xho*I end and a blunt *Eco*RV half-site upon annealing. These three fragments were ligated into *Sac*I-linearized pBluescript+ (Stratagene, La Jolla, Calif.) to generate p6UL8.

(iv) UL52 constructs. The UL52 ORF was isolated on an *Xba*I fragment from pCM-UL52 (17) and inserted into the *Xba*I site of pGEM-3Zf$^+$ (Promega) to generate pGM3/UL52. The UL52 ORF was placed under control of the ICP6 promoter as follows. The *Xba*I fragment from pCM-UL52 was inserted into the *Xba*I site in the pUC119 polylinker to generate p119UL52. The ICP6 promoter was isolated on a *Hin*dIII-to-*Xho*I fragment from pDG-2 (39) and inserted into p119UL52 digested with *Hin*dIII and *Sal*I. The resulting plasmid, which contains the UL52 gene under the control of the ICP6 promoter, is designated p6UL52.

Construction of epitope-tagged UL5, UL8, and UL52. Plasmids capable of expressing the helicase-primase complex genes fused to short defined peptide epitopes from the ICP6 promoter were constructed as described below. Sequences encoding the epitopes are double underlined. Correct insertion of each epitope was confirmed by DNA sequence analysis.

(i) Construction of AU1-UL5. A fragment containing UL5 sequences upstream of the *Rma*I site (15106) was isolated on an *Rma*I-to-*Xho*I fragment from pBamUL5b. This was ligated in frame to an annealed pair of complementary synthetic oligonucleotides containing a sequence coding for an N-terminal AU1 epitope (DTYRYI) fused to the sequence of UL5 upstream of the *Rma*I site (15133 to 15106). The oligonucleotide 5′-GATCCGCCACCATGGACACCTA TCGCTATATA**ATG**GCGGCGGCCGGCGGGGAAGCGCCAGC-3′ and its complement were designed to yield cohesive *Bam*HI and *Rma*I sites upon annealing. The resulting *Bam*HI-to-*Xho*I fragment was ligated into *Bam*HI-*Xho*I-digested p6UL5119b. The *Bam*HI-to-*Sac*II (13277) fragment containing the AU1-tagged N-terminal portion of UL5 was excised and ligated to the *Sac*II-to-*Bam*HI fragment of p6UL5119a to regenerate the complete UL5 ORF. This *Bam*HI fragment was inserted into the expression vector p6NBam at the *Bam*HI site to generate p6AU1UL5 for expression from the ICP6 promoter.

(ii) Construction of UL52-KT3. PCR was used to introduce the KT3 peptide epitope (TEPEPPPT) onto the C terminus of UL52. The forward primer began 57 bases upstream of the *Nru*I site within the UL52 ORF and had the sequence 5′-ACGACGAGTTGCCTACTTTG-3′ (111250 to 111230). The reverse primer had the sequence 5′-GTGTGTGGATCCACGCCCGTTA**TCA**TGTTTCTGGT TCTGGTGGTGGTGTAGACGACGGTTG-3′ and introduced the KT3 epitope onto the C terminus of UL52 and a *Bam*HI site downstream of the stop codon. The resulting 975-bp product was digested with *Nru*I and *Bam*HI and cloned into the superlinker plasmid pSL301 (Invitrogen, San Diego, Calif.) to generate pSL301UL52KT3. The *Nru*I-to-*Bam*HI fragment from this construct was used in a three-way ligation with a *Bam*HI-*Nru*I fragment from pGM3/52, which contains the rest of the UL52 gene, and a vector digested with *Bam*HI that contains the ICP6 promoter (p6NBam). This construct is designated p6UL52KT3.

(iii) Construction of EE-UL8. UL8 sequences downstream of the *Eco*RV (20463) site were isolated on an *Eco*RV-to-*Bam*HI fragment from pGM7/UL8. This fragment was ligated to a set of annealed synthetic complementary oligonucleotides containing sequences coding for an N-terminal EE epitope (EE YMPME) and the amino acids of UL8 upstream of the *Eco*RV site (20463 to 20478). The oligonucleotide 5′-GGATCCTTACC**ATG**GAAGAATATATGC CAATGGAAGACACCGCAGAT-3′ and its complement were designed to yield a cohesive *Bam*HI site and a blunt *Eco*RV half-site upon annealing. The resulting *Bam*HI fragment was inserted into the *Bam*HI site of p6NBam to generate p6EEUL8.

Transient complementation assay. A total of 1.5×10^6 Vero cells were transfected with 12 μg of native or epitope-tagged UL5-, UL8-, and UL52-containing constructs or were mock transfected by a modification of the standard calcium phosphate precipitation procedure (15). Cells were superinfected with 1 PFU of the appropriate virus per cell 18 to 20 h posttransfection. Virus was adsorbed for 1 h, and the innoculum was aspirated and replaced with growth medium. Infected cells were incubated for 18 h, harvested (including the growth medium), frozen

TABLE 2. Complementation of *hr*99, *hr*80, and *hr*114 with native and epitope-tagged constructs[a]

Plasmid	Superinfecting virus	Complementation index[b]
p6UL5	*hr*99	176
p6AU1UL5	*hr*99	196
p6UL8	*hr*80	672
p6EEUL8	*hr*80	124
p6UL52	*hr*114	225
p6UL52KT3	*hr*114	93

[a] Vero cells were transfected with the indicated plasmid and superinfected with the indicated virus. The results of two experiments were averaged.

[b] Complementation index for each virus is determined as the PFU of the null mutant from cells transfected with the indicated plasmid/PFU of the null mutant from mock-transfected cells.

at −70°C, thawed rapidly at 37°C, and sonicated for 1 min on ice. The supernatants were clarified, and the titers on permissive cells were determined as follows: *hr*99 on the UL5-expressing cell line L2-5 (39), *hr*80 on the UL8-expressing cell line SL8 6-6 (32a), and *hr*114 on the UL52-expressing cell line BL-1 (13).

Indirect immunofluorescence. For transfection-superinfection experiments, 1.0×10^6 Vero cells were transfected with 8 μg (total) of plasmid DNA as described above for the transient complementation assay. Cells were grown on coverslips, adsorbed for 1 h with 10 to 20 PFU of the desired virus per cell, and incubated for 5.5 h postadsorption in the presence or absence of ACG (100 μM) or PAA (400 μg/ml). When indicated, cells were labeled with 1 mM BrdU for 15 min prior to fixation. Cells were fixed in 37% buffered formaldehyde (Sigma) diluted 1:10 in phosphate-buffered saline (PBS [pH 7.4]) for 30 min and permeabilized with 1.0% Triton X-100 for 10 min. If cells had been labeled with BrdU, they were treated with 4 M HCl for 10 min. Cells were reacted with primary antibodies in 3% normal goat serum in PBS for 30 min. Anti-AU1 ascites were used at 1:1,000, anti-EE ascites were used at 1:500, anti-KT3 ascites were used at 1:500, anti-BrdU antibodies were used at 1:50, and 3-83 was used at 1:1,000 as indicated. Cells were reacted with secondary antibodies in 3% normal goat serum in PBS at a 1:200 dilution for 30 min. Coverslips were mounted in glycerol gelatin containing 2.5% DABCO to retard bleaching.

Imaging. Cells stained for immunofluorescence were imaged on a Zeiss Axiovert 135 laser scanning microscope (confocal) equipped with a Zeiss ×630 Plan Neofluar objective. Collected images were arranged and labeled with a Silicon Graphics Work station equipped with Adobe Photoshop 3.0.

RESULTS

Constructs encoding epitope-tagged UL5, UL8, and UL52 produce stable, functional proteins. To facilitate studies of the localization of the helicase-primase complex subunits during infection, we added sequences to each subunit that encode peptide epitopes which can be recognized by monoclonal antibodies. To confirm that these constructs produced stable proteins that could be detected immunologically, Vero cells were transfected with expression clones, superinfected, and processed for Western blot (immunoblot) analysis. Each of the constructs produces stable protein that is of the expected molecular weight and which is recognized both by the monoclonal antibody recognizing its epitope tag and by its cognate polyclonal antibody (data not shown).

Each of the epitope-tagged proteins was found to be functional by two approaches. First, the tagged proteins were capable of complementing their respective null mutant viruses for replication of an HSV-1 origin-containing plasmid (data not shown). Second, each of the tagged constructs was tested in a transient complementation assay. This assay assesses the ability of the epitope-tagged proteins, expressed from the HSV-1-inducible ICP6 promoter on a transfected plasmid, to complement the yield of its cognate null virus. Vero cells were transfected with plasmids which express either the native or tagged version of each gene (UL5, UL8, or UL52) and subsequently were superinfected with their cognate null mutant viruses (Table 2). All constructs complemented their respective null mutant viruses efficiently. The biological significance of the differences in complementation indices between the tagged and native proteins is unclear. We cannot rule out the possibility that the addition of the epitope tags to the proteins might subtly affect overall virus yields. However, it appears that each is functional for the complementation of its cognate null mutant virus.

Localization of epitope-tagged UL5, UL8, and UL52 in infected cell nuclei. UL29, UL30, UL42, and UL9 all localize to replication compartments (14, 30, 31). Olivo et al. were unable to detect a subnuclear localization of UL5, UL8, and UL52 with polyclonal antisera in an indirect immunofluorescence assay, perhaps because of the low sensitivity of the antisera they used (30). We reasoned that the members of the helicase-primase complex should localize to these sites of viral DNA synthesis since they are essential replication proteins. To test this hypothesis, the epitope-tagged constructs described above were used in an indirect immunofluorescence assay to examine the localization of the helicase-primase complex in infected nuclei.

Previous studies suggested that efficient translocation of the helicase-primase complex into the nucleus required coexpression of all of its subunits (3). Indeed, Vero cells that were transfected with p6AU1UL5 alone and superinfected with *hr*99 exhibited extensive cytoplasmic staining for the AU1 epitope in an immunofluorescence assay (data not shown). Accordingly, transfection experiments were designed to ensure that each of the helicase-primase complex subunits was expressed to a similar level. For instance, in order to study the localization of AU1-UL5 in infected nuclei, cells were cotransfected with p6AU1UL5, p6UL8, and p6UL52 and superinfected with *hr*99. Cells on coverslips were stained for immunofluorescence with the polyclonal antiserum 3-83, which recognizes UL29, and a monoclonal antibody which recognizes the AU1 epitope of AU1-UL5 as described in Materials and Methods. Figure 1B shows that UL29 localizes to typical replication compartments. Figure 1A shows the AU1 staining pattern in the same cell. The concordant patterns of staining demonstrate that AU1-UL5 and UL29 colocalize in replication compartments. Within the large globular replication compartments, substructures can be seen as previously reported for UL29 (8). EE-UL8 and UL52-KT3 were also shown to colocalize with UL29 in replication compartments in a similar manner. Figure 1C and D illustrate the colocalization of EE-UL8 and of UL29, respectively. Figure 1E and F demonstrate that UL52-KT3 and UL29, respectively, colocalize in replication compartments.

To determine if the members of the helicase-primase complex also colocalize with UL29 in prereplicative sites, these experiments were repeated in the presence of PAA. Each of the tagged proteins colocalizes with UL29 in prereplicative sites (Fig. 2). Prereplicative sites are distributed throughout the nucleus, whereas replication compartments appear to be restricted in their distribution (compare Fig. 1 and 2). To ensure that the epitope tag staining was not simply spurious signal resulting from the overflow of fluorescein emissions into the Texas red channel, cells were also stained with the monoclonal antibodies alone. The staining pattern for each was typical of prereplicative sites (data not shown). In addition, colocalization was also observed when the secondary antibody fluors were switched (data not shown). In summary, the epitope-tagged proteins proved to be useful reagents for demonstrating that UL5, UL8, and UL52 colocalize with UL29 both in replication compartments and in prereplicative sites. Furthermore, this is the first demonstration of helicase-primase complex localization to replication compartments and prereplicative sites.

Prereplicative site formation in viral mutants which fail to express UL5, UL8, UL52, or UL9. UL29 is essential for pre-

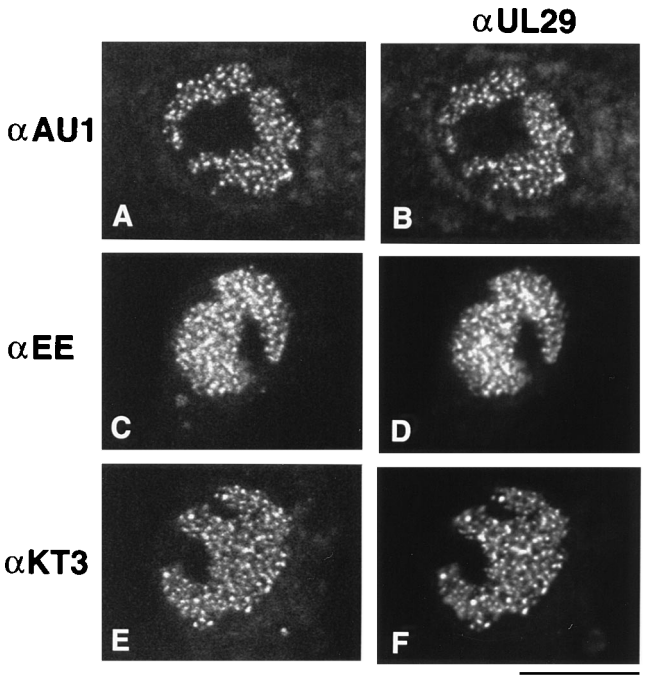

FIG. 1. Cotransfected-superinfected cells doubly labeled with 3-83 and monoclonal antibodies recognizing epitope-tagged UL5, UL8, and UL52. Vero cells were cotransfected and superinfected as described in Materials and Methods. Cells were fixed and permeabilized at 5.5 h postsuperinfection as described in Materials and Methods. (A and B) Vero cells were cotransfected with p6AU1UL5, p6UL8, and p6UL52 and superinfected with *hr*99. (C and D) Vero cells were cotransfected with p6UL5, p6EEUL8, and p6UL52 and superinfected with *hr*80. (E and F) Vero cells were cotransfected with p6UL5, p6UL8, and p6UL52KT3 and superinfected with *hr*114. (A, C, and E) Staining for rhodamine-conjugated goat anti-mouse secondary antibodies (α) reacting with, respectively, AU1 ascites, EE ascites, and KT3 ascites. (B, D, and F) Staining pattern of 3-83 as recognized by fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody in the same cells. Marker bar, 15 μm.

replicative site formation (7). However, it is not sufficient for their formation; UL29 is diffusely distributed in cell nuclei transfected with a UL29 expression clone (10) (data not shown). We hypothesize that other essential replication proteins may be necessary for prereplicative site formation. In order to determine if the helicase-primase subunits or UL9 was essential for prereplicative site formation, cells infected with *hr*80, *hr*99, *hr*114, or *hr*94 (UL8, UL5, UL52, and UL9 null mutant viruses, respectively) were examined for UL29 staining by indirect immunofluorescence. In cells infected with *hr*80, *hr*99, or *hr*114, UL29 staining was diffuse and nuclear (Fig. 3A, panels A, D, and G). When the infections were carried out in the presence of PAA, greater than 50% of the cells contained what appeared to be prereplicative sites (Fig. 3A, panels B, E, and H; Fig. 3B, panel A). To demonstrate that the formation of prereplicative sites was not due to an unusual effect of PAA treatment, UL29 staining patterns were also examined in the presence of ACG, another specific inhibitor of HSV polymerase. Similar results were obtained (Fig. 3A, panels C, F, and I). Cells infected with *hr*94 behaved like cells infected with the helicase-primase null mutants (described below). Thus, UL29 is distributed diffusely in the nuclei of cells infected with viruses that fail to express UL5, UL8, UL52, or UL9. In the presence of PAA or ACG, however, UL29 localizes to prereplicative sites in cells infected with these mutants.

One of the characteristics of prereplicative sites formed in drug-treated KOS-infected cells is that UL29 staining colocalizes with incorporated BrdU at sites thought to represent areas of cellular replication (7). To determine whether the prereplicative sites described above also stain with BrdU, mutant-infected cells were treated with BrdU for 15 min prior to fixation and were doubly stained with a monoclonal antibody which recognizes BrdU and with 3-83. In Fig. 3B, panels A and B show the BrdU and 3-83 staining patterns, respectively, in a group of cells infected with *hr*94 in the presence of PAA. Although three cells are visible in Fig. 3, two exhibit diffuse nuclear staining for UL29 and no BrdU staining, and one exhibits colocalization of UL29 and BrdU incorporation in prereplicative sites. Similar results were observed in KOS- and the other null mutant-infected cells (data not shown). Thus UL29 exhibits a prereplicative site staining pattern only in cells which also incorporate BrdU. This is in contrast with previous studies which concluded that some proportion of KOS-infected cells treated with PAA contained prereplicative sites but no BrdU staining (7). This discrepancy may reflect a difference in staining conditions.

A polymerase null mutant virus forms prereplicative sites in the absence of PAA. Prereplicative sites can be found in the nuclei of cells infected with temperature-sensitive UL30 or temperature-sensitive UL42 mutants at the nonpermissive temperature or with a null mutant in UL30 (2, 14, 31). Thus, inactivation of the polymerase complex with drugs or by means of mutation leads to prereplicative site formation. To determine whether the prereplicative sites observed in cells infected with a polymerase null mutant also stained with BrdU, Vero cells were infected with HP66, which contains a deletion/insertion mutation in UL30, and stained with 3-83 and BrdU. Prereplicative sites were present in both the absence (Fig. 4A,

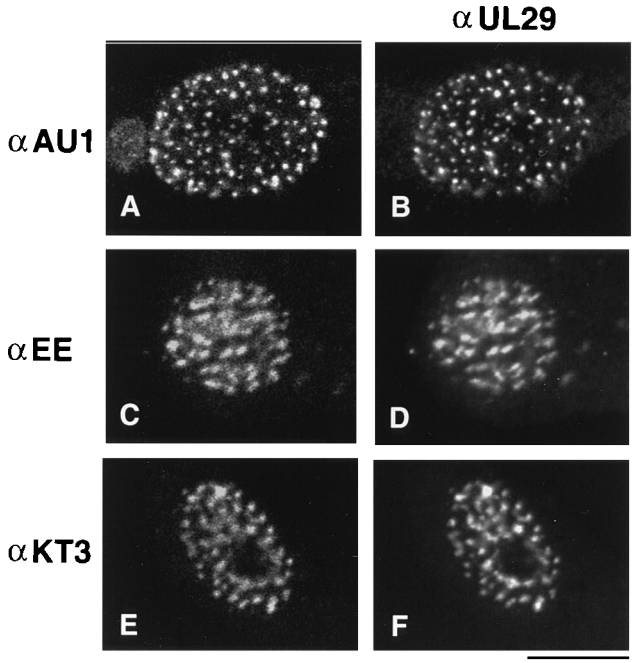

FIG. 2. Cotransfected-superinfected cells grown in the presence of PAA and doubly labeled with 3-83 and monoclonal antibodies recognizing epitope-tagged UL5, UL8, and UL52. Cells were cotransfected, superinfected, and processed as described in the legend to Fig. 1, except that superinfection was carried out in the presence of 400 μg of PAA per ml. (A, C, and E) Staining for Texas red-conjugated goat anti-mouse secondary antibodies (α) reacting with, respectively, AU1 ascites, EE ascites, and KT3 ascites. (B, D, and F) Staining pattern of 3-83 as recognized by fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody in the same cells. Marker bar, 15 μm.

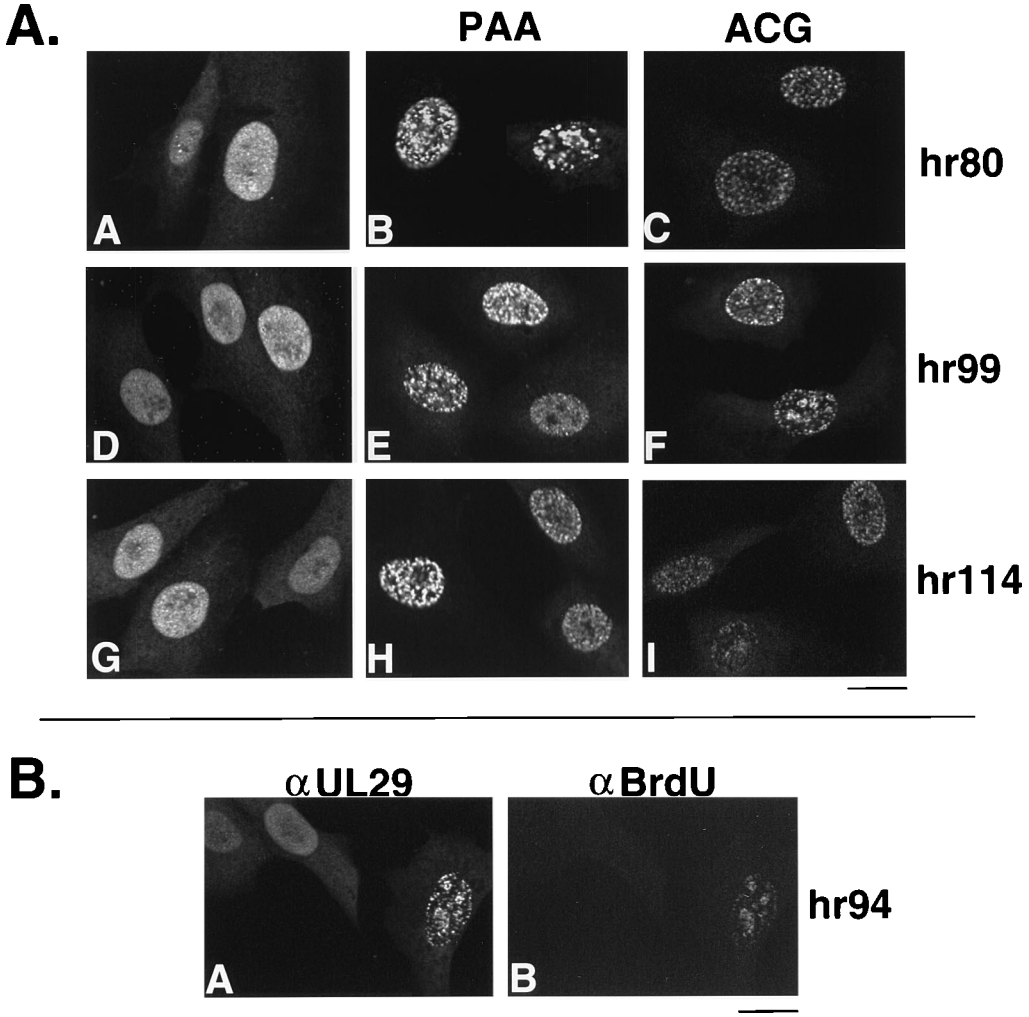

FIG. 3. UL29 localization in cells infected with viral mutants that fail to express UL5, UL8, UL52, or UL9. Vero cells were infected with 20 PFU of virus per cell and processed as described in Materials and Methods. (A) Cells were stained with 3-83 and fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody. Cells were infected with the following mutants: panels A, B, and C, *hr*80; D, E, and F, *hr*99; G, H, and I, *hr*114. Infected cells in panels B, E, and H were treated with PAA, and those in panels C, F, and I were treated with ACG. Marker bar, 15 μm. (B) *hr*94-infected cell doubly stained with antibodies (α) for UL29 and BrdU. Panels A and B show the same group of *hr*94-infected cells doubly stained, respectively, with 3-83 (fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody) and anti-BrdU (Texas red-conjugated goat anti-mouse secondary antibody). Marker bar, 15 μm.

panel A) and the presence (Fig. 4A, panel B) of PAA. BrdU and UL29 colocalize in these sites (Fig. 4B, panels A and B, respectively). This is the first demonstration of UL29 and BrdU colocalization in cells infected with a polymerase null mutant. We therefore conclude, in agreement with Bush et al. (2), that the polymerase polypeptide itself is not necessary for prereplicative site formation.

Viruses containing inactivating point mutations in UL5 and UL9 helicase motifs fail to form prereplicative sites in the absence of PAA. The results presented above suggest that inhibition of the viral DNA polymerase results in the formation of prereplicative sites. To determine if the inhibition of HSV replication by other methods which do not involve the absence of an essential replication protein also results in prereplicative site formation, a number of viral mutants with subtle mutations in UL5 or UL9 were examined. The UL5 gene contains six well-conserved helicase motifs. Single-amino-acid substitutions of highly conserved residues in each of the motifs result in the loss of function of the UL5 protein as assessed in an in vivo replication complementation assay (38). Three of these mutations were introduced into the HSV-1 genome, and the resulting viruses are replication defective: *hr*99G102V and *hr*99K103A contain single-amino-acid substitutions in helicase motif I, and *hr*99R345K contains a single-amino-acid substitution in helicase motif IV (38). These mutants produce stable mutant UL5 proteins that are capable of interacting with UL8 and UL52 in an immunoprecipitation assay, implying that their global conformation is not grossly altered (38).

Vero cells infected with *hr*99G102V exhibit a diffuse nuclear staining for UL29 (Fig. 5A), whereas *hr*99G102V-infected cells treated with PAA exhibit a staining pattern for UL29 typical of prereplicative sites (Fig. 5B). Cells infected with *hr*99K103A and *hr*99R345K behave identically (data not shown). Similar experiments were carried out with *hr*27, a viral mutant which contains two amino acid substitutions in the conserved helicase domain IV of UL9 (21). This mutant is replication defective but produces a UL9 protein with the predicted molecular weight. Vero cells infected with *hr*27 in the absence of PAA show a diffuse nuclear staining for UL29 (Fig. 5C). However, treatment of *hr*27-infected cells with PAA results in the local-

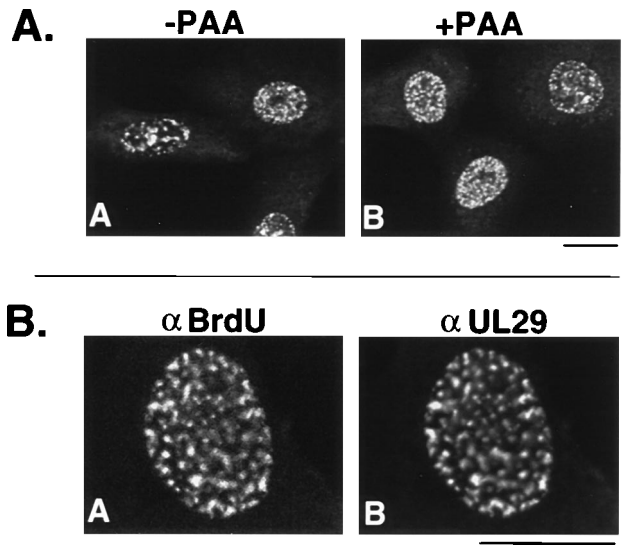

FIG. 4. UL29 localization in HP66-infected cells. Vero cells were infected with 20 PFU of virus per cell and processed as described in Materials and Methods. (A) Panels A and B show 3-83 staining as detected by fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody in cells infected in the presence (+) and absence (−) of PAA, respectively. Marker bar, 15 μm. (B) HP66-infected cell doubly stained with antibodies (α) for BrdU and UL29. Panels A and B show a cell nucleus doubly stained, respectively, with anti-BrdU (Texas red-conjugated goat anti-mouse secondary antibody) and 3-83 (fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody). Marker bar, 15 μm.

ization of UL29 to prereplicative sites in greater than 50% of infected nuclei (Fig. 5D). ACG treatment of cells infected with the UL5 or UL9 helicase motif mutants resulted in prereplicative site formation in a similar proportion of cells (data not shown). In the presence of either drug, prereplicative sites colabel with BrdU. We conclude that inhibition of viral DNA synthesis by subtle mutation of UL5 or UL9 is not sufficient to induce prereplicative site formation unless the viral polymerase is also inhibited.

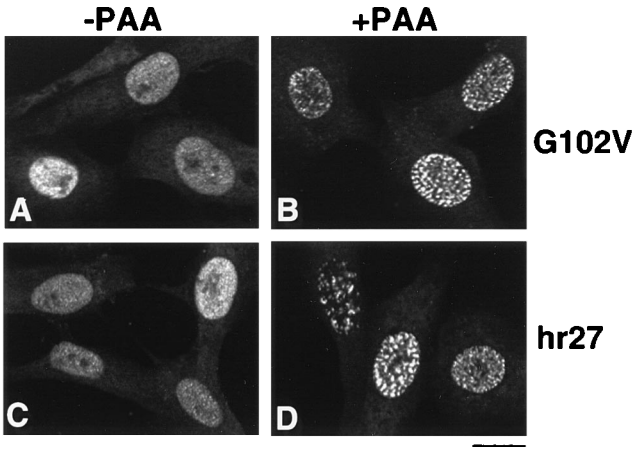

FIG. 5. UL29 localization in cells infected with *hr*99G102V or *hr*27. Vero cells were infected with 20 PFU of *hr*99G102V (A and B) or *hr*27 (C and D) per cell and processed as described in Materials and Methods. Cells were stained with 3-83 and fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody. Infected cells in panels B and D were treated with PAA, while those in panels A and B were not. Marker bar, 15 μm.

TABLE 3. Prereplicative site formation in replication-defective viruses

Virus	Prereplicative site formation	
	−PAA	+PAA
KOS	RC[a]	+
*hr*99	−	+
*hr*99G102V	−	+
*hr*99K103A	−	+
*hr*99R345K	−	+
*hr*80	−	+
*hr*94	−	+
*hr*27	−	+
*hr*114	−	+
HP66	+	+

[a] RC, forms replication compartments.

DISCUSSION

Using epitope-tagged versions of UL5, UL8, and UL52, we have demonstrated that the helicase-primase complex colocalizes with UL29 in replication compartments and prereplicative sites in HSV-1-infected nuclei. With this observation, all seven of the essential replication genes have now been shown to be present in replication compartments. To determine if the helicase-primase complex and UL9 play a role in prereplicative site formation, we examined UL29 staining patterns in cells infected with various replication-defective viruses (see Table 3 for summary). The UL29 staining pattern of cells infected with null mutants of UL5, UL8, UL9, and UL52 is diffuse nuclear. In the presence of PAA and ACG, prereplicative sites exhibiting colocalized UL29 and BrdU staining were observed in greater than 50% of the cells. The remaining cells exhibit diffuse UL29 staining and no BrdU incorporation. One explanation for the observation that prereplicative sites are not seen in 100% of infected cells is that prereplicative site formation requires cells to be competent to carry out cellular DNA synthesis. This may imply a requirement for a natural or induced progression into S phase, although we cannot rule out that BrdU incorporation is not due to repair synthesis.

The fact that prereplicative sites can form in mutant-infected cells treated with PAA or ACG suggests that their formation may not require the UL5, UL8, UL52, or UL9 proteins. These observations are inconsistent with the idea that prereplicative sites represent intermediates in the assembly of the viral replication apparatus. However, it is possible that viral polymerase inhibitors alter the cellular milieu such that prereplicative sites can form in the absence of viral factors that would normally be necessary. Under this scenario, the fact that UL29 does not localize to prereplicative sites in cells infected with *hr*80, *hr*99, *hr*114, and *hr*94 unless polymerase inhibitors are added suggests that these four replication proteins are essential for prereplicative site formation (19a). However, our observation that prereplicative sites do not form in cells infected with subtle motif mutants of UL5 and UL9 in the absence of polymerase inhibitors implies that these viral proteins are probably not essential for their formation.

A more likely explanation for these results is that an active viral polymerase complex prevents or inhibits prereplicative site formation. Several mechanisms can be considered. Active polymerase may affect or interact with some factor, viral or cellular, which is necessary for prereplicative site formation. An attractive candidate factor is UL29, which is essential for prereplicative site formation (2, 7). Perhaps an active polymerase complex prevents UL29 from acting as a focus for

prereplicative site formation or acts to disrupt these sites. Specifically, active viral polymerase may remove UL29 that is associated with cellular single-stranded DNA. Our observation that UL29 in prereplicative sites is always associated with cellular DNA replication lends indirect support to the concept that UL29 may be bound to single-stranded DNA. Accordingly, when the polymerase complex is inactivated, UL29 would remain bound to single-stranded DNA, thereby forming prereplicative sites. Interestingly, there is genetic evidence for an interaction between UL29 and the polymerase complex (5).

Clearly, all of the factors and conditions necessary for prereplicative site formation have yet to be identified. Cells transfected with UL29 alone only rarely display nuclear structure when examined with anti-UL29 antibodies (11). In contrast, nuclear structures form in cells cotransfected with UL5, UL8, UL52, and UL29, which resemble prereplicative sites (unpublished observations). This suggests that UL5, UL8, and UL52 can mediate UL29 localization in transfected cells. This observation may seem contradictory to the model presented above, which suggests that UL5, UL8, and UL52 cannot be considered necessary for prereplicative site formation. However, prereplicative sites may form in two or more different ways. It is possible that in the absence of an active helicase-primase complex, another protein may be capable of mediating prereplicative site formation. The presence of this "other factor" would explain the formation of prereplicative sites in *hr*80-, *hr*99-, and *hr*114-infected cells. One attractive candidate for the other factor is UL9. Like UL5 in the helicase-primase complex, UL9 contains conserved helicase motifs that are essential for its ability to catalyze origin-dependent DNA replication in vivo (23). Furthermore, UL9 has been shown to directly interact with UL29 (1). Perhaps either one of the HSV-1 helicases, UL5 in the helicase-primase complex or UL9, could independently mediate the localization of UL29 to prereplicative sites. The putative requirement for one active helicase may reflect the need to expose single-stranded DNA or may reflect conformational changes which occur upon interaction between various members of the DNA synthetic machinery.

The possibility that cellular factors may play a role in prereplicative site formation and replication compartment formation should also be considered. Wilcock and Lane have demonstrated that a number of host replication proteins, as such as proliferating cell nuclear antigen and DNA polymerase α, colocalize with UL29 both in prereplicative sites and in replication compartments (36). Currently, there is no in vitro system for HSV-1 genome replication. This may indicate the need for host factors in addition to the essential viral replication proteins. Until other factors are definitively identified that participate in prereplicative site formation, it will be difficult to conclusively determine whether or not they represent biologically relevant subassemblies of replication proteins. Nevertheless, the study of these structures in infected cells and transfection systems may prove to be a powerful tool for understanding the process of HSV-1 DNA replication.

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