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Herpes Simplex Virus Type 1 Prereplicative Sites Are a Heterogeneous Population: Only a Subset Are Likely To Be Precursors to Replication Compartments

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When herpes simplex virus type 1 (HSV-1) DNA replication is blocked by viral polymerase inhibitors, such as phosphonoacetic acid (PAA) or acyclovir (ACV), UL29 (ICP8) localizes to numerous punctate nuclear foci which are called prereplicative sites. Since this pattern can form in cells infected with mutants which are defective in UL5, UL8, UL9, or UL52 in the presence of polymerase inhibitors (C. J. Lukonis and S. K. Weller, J. Virol. 70:1751–1758, 1996; L. M. Liptak, S. L. Uprichard, and D. M. Knipe, J. Virol. 70:1759–1767, 1996), we previously proposed that it is unlikely that these numerous UL29 foci actually represent a functional subassembly of viral replication proteins that could lead to the formation of replication compartments (C. J. Lukonis and S. K. Weller, J. Virol. 70:1751–1758, 1996). In this paper, we have investigated the requirement for formation of the prereplicative site pattern by using double mutants of HSV. From the analysis of mutants lacking both UL5 and UL9, we conclude that neither viral helicase is required for the prereplicative site pattern to form as long as a polymerase inhibitor is present. From the analysis of mutants defective in both UL30 and UL5, we suggest that the prereplicative site pattern can form under conditions in which viral and/or cellular polymerases are inhibited. Furthermore, reexamination of the UL29 staining pattern in cells infected with wild-type virus in the presence of PAA reveals that at least two different UL29 staining patterns can be detected in these cells. One population of cells contains numerous (greater than 20) punctate UL29 foci which are sites of cellular DNA synthesis. In another population of cells, fewer punctate foci (less than 15) are detected, and these structures do not colocalize with sites of cellular DNA synthesis. Instead, they colocalize with PML, a component of nuclear matrix structures known as ND10. We propose that ND10-associated UL29 sites represent domains at which replication compartments form.

Herpes simplex virus type 1 (HSV-1) is a large double-stranded DNA virus which replicates in the nuclei of infected cells. Seven viral genes are essential for HSV-1 DNA replication, including 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 19, 40, and 53). HSV DNA replication takes place in globular nuclear structures termed replication compartments (6, 42, 46), and all seven HSV-1 replication proteins colocalize within these structures (15, 24, 26, 29, 41, 42, 46). The observation that replication compartments are present in nearly identical patterns in binucleate cells suggests that preexisting nuclear architecture, perhaps the nuclear matrix, determines the organization of replication compartments in an infected cell (7). A possible explanation for the relationship between replication compartments and the nuclear matrix comes from the recent finding that input viral DNA is deposited in close proximity to nuclear domains named ND10 (18). These nuclear matrix-bound domains are present in most cell types at an average frequency of 10 (1, 34). Furthermore, replication compartments form at the periphery of ND10 and remain associated with that portion of the nuclear matrix during infection (35). Among the many proteins that are associated with ND10 is the tumor suppressor PML (1, 9, 21, 22, 39, 52). Although the function of ND10 is not known, these structures may play a role in cellular processes such as gene expression and/or proliferation. HSV infection causes the disruption of ND10, and the viral protein primarily responsible for the disruption is ICP0 (12, 33, 34). The ability of ICP0 to alter nuclear substructure may be critical for the efficient progression of a productive infection by providing an atmosphere conducive to viral transcription and replication, because viral mutants in the ICP0 gene, which are unable to disrupt ND10, are compromised for growth at low multiplicities of infection (MOI) (3, 47, 50).

We have recently demonstrated that replication compartments can be formed by cotransfecting Vero cells with constructs expressing the seven essential viral replication proteins and a plasmid containing an HSV-1 origin of DNA replication. Like replication compartments in infected cells, these compartments contain all of the essential viral replication proteins, are sites of DNA synthesis, and are found at the periphery of ND10 (27). Additionally, we showed that UL29 alone localizes adjacent to ND10 in transfected Vero cells (27). Thus, UL29 may direct replication compartment formation at predetermined sites in the nucleus.

When viral replication is blocked by viral polymerase inhibitors, such as phosphonoacetic acid (PAA) or acyclovir (ACV), UL29 localizes to numerous punctate foci, which are called prereplicative sites, many of which colabel with bromodeoxyuridine (BrdU) (6). Since viral DNA synthesis is inhibited, it is likely that these sites of BrdU staining represent de novo replication of cellular DNA by a cellular polymerase; in fact, de Bruyn Kops and Knipe defined prereplicative sites based on the presence of cellular DNA synthesis (6). Several HSV-1 replication proteins (UL5, UL8, UL29, UL30, and UL52) also

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colocalize at these numerous sites of UL29 staining (2, 15, 24, 26); however, UL9 and UL42 do not (2, 15, 29). At very early times after infection, UL29 is localized to structures in the nucleus which resemble prereplicative sites (6), and it has been suggested that structures similar or equivalent to prereplicative sites may represent intermediates in the assembly of viral replication compartments (6, 24). However, the observation (24, 26) that the prereplicative site pattern can form in mutants defective for the essential replication proteins UL5, UL8, UL9, and UL52 under certain conditions has led to confusion over whether all UL29 foci which are found in the prereplicative site pattern represent intermediates in the formation of replication compartments (26).

This study was undertaken to examine the nature of prereplicative sites and to establish the requirements for their formation. We have used double mutant viruses to establish conditions under which the prereplicative pattern of UL29 staining can be observed. We have also examined cells infected with wild-type HSV-1 in the presence of polymerase inhibitors. In some PAA-treated cells, numerous (generally more than 20) punctate foci are detected which primarily colocalize with BrdU. In other PAA-treated cells, fewer punctate foci are detected, and these structures do not colocalize with BrdU; however, they do colocalize with ND10 antigen. We propose that the ND10-associated UL29 structures are likely to represent sites at which replication compartments form. This interpretation is consistent with the observation that input viral DNA is deposited at ND10 and that replication compartments form at the periphery of ND10 (18, 35). We further postulate that the numerous UL29 foci seen in some infected cells do not represent intermediates in the assembly of viral replication compartments; instead, they likely represent areas of UL29 accumulation at sites of exposed single-stranded DNA. Models for how these areas of single-stranded DNA might be created will be discussed. We conclude that the previously designated prereplicative site pattern actually encompasses a heterogeneous mixture of patterns. Combined with previous results indicating that UL29 expressed by itself localizes to ND10 (27), the results in this paper indicate that UL29 may play a key role in localizing replication compartments to ND10.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney fibroblasts (Vero; American Type Culture Collection) and HEp2 cells (American Type Culture Collection) were propagated and maintained as described previously (54). Cell lines containing one or more HSV-1 genes were used as complementing cell lines. G418-resistant 2B11 cells containing the UL9 gene under the control of the strong inducible HSV-1 ICP6 promoter (28), G418-resistant L2-5 cells containing the UL5 gene under control of the ICP6 promoter (56), and G418-resistant B3 cells containing the HSV-1 UL30 gene were generously provided by C. B. C. Hwang (SUNY Heath Science Center, Syracuse, N.Y.). The G418/hygromycin-resistant DTMA cell line containing the UL5 and the UL30 genes and the G418/hygromycin-resistant Hel28 cell line containing the UL5 and UL9 genes were constructed as part of this study (see below). G418-resistant cell lines were treated with medium (Dulbecco's modified Eagle's medium plus 5% fetal calf serum) containing 500 μg of G418 (Geneticin; GIBCO Laboratories, Grand Island, N.Y.) per ml approximately every fifth passage. G418/hygromycin-resistant cell lines were treated with medium containing 400 μg of G418 per ml and 70 to 90 μg of hygromycin B (Sigma, St. Louis, Mo.) per ml approximately every fifth passage.

The KOS strain of HSV-1 was used as the wild-type virus. *hr*94 is a UL9 null mutant virus containing an ICP6::*lacZ* insertion and was propagated on 2B11 cells (28); *hr*99, a UL5 null mutant virus containing an ICP6::*lacZ* insertion, and *hr*99R345K, containing a single point mutation in UL5 helicase motif IV, were propagated on L2-5 cells (57). The variant protein encoded by the R345K UL5 mutant is defective in helicase activity (16). HP66 is a UL30 null mutant virus and was kindly provided by Donald M. Coen (Harvard Medical School, Boston, Mass.) and was propagated on B3 cells (31). Double mutant viruses are described in Results.

Reagents and antibodies. Glycerol gelatin, 1,4-diazobicyclo-[2.2.2]octane (DABCO), PAA, and ACV were obtained from Sigma. BrdU was obtained from Boehringer Mannheim (Mannheim, Germany). The polyclonal antiserum 3-83, which recognizes UL29, was a generous gift of David Knipe (Harvard Medical School) (20). The polyclonal antiserum α-ICP8, which recognizes UL29 used in the colocalization experiments, was a generous gift of Bill Ruyechan (SUNY at Buffalo) (48). The monoclonal antibody that recognizes BrdU was obtained from Becton Dickinson Immunocytometry Systems (San Jose, Calif.). The monoclonal antibody that recognizes PML was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse antibodies were obtained from Cappel, Organon Teknika Corporation (Durham, N.C.).

Plasmids. p6UL5119b, containing the UL5 gene under the control of the ICP6 promoter, was described previously (26). pCM-HM, containing the hygromycin phosphotransferase gene under the control of the cytomegalovirus immediate-early promoter, was generously provided by Robert Harrington (University of Connecticut Health Center).

Isolation of cell lines. Cell lines permissive for the propagation of mutants lacking UL30 and UL5 were isolated by stable cotransfection of a construct expressing UL5 (p6UL5119b) and a selectable hygromycin resistance marker (pCM-HM) into the B3 cell line as previously described (57), except that hygromycin B was used to select resistant cells at a concentration of 100 μg of hygromycin B per ml. After most of the cells had died, the concentration of hygromycin B was lowered to 60 to 75 μg/ml. Colonies of cells resistant to hygromycin B and G418 were picked and propagated in Dulbecco's modified Eagle's medium supplemented with 60 μg of hygromycin per ml and 250 μg of G418 per ml. Immunofluorescence (IF) microscopy was used to screen for cell lines that could complement both *hr*99 and HP66 mutants as described in Results. A cell line expressing UL5 and UL9 was generated by stable cotransfection of p6UL5119b and pCM-HM into the 2B11 cell line by the same procedures described above for the UL5/UL30 cell line.

Analysis of viral DNA synthesis. Viral DNA synthesis was analyzed by the dot blot method, essentially as described previously (57). Briefly, 1.5×10^6 cells were infected with virus at an MOI of 10 PFU/cell and incubated for 18 to 24 h. Cells were scraped into 15-ml Falcon tubes, precipitated by centrifugation at 1,500 rpm for 2 to 5 min in a Fisher microcentrifuge (model 235B), resuspended in Tris-buffered saline, and centrifuged twice. Cell pellets were resuspended in 200 μl of phosphate-buffered saline (PBS). Eighty microliters and four fivefold dilutions of the suspension were spotted onto a GeneScreen Plus membrane by using a Microsample Filtration Manifold (Schleicher and Schuell). The membrane was dried, treated twice with 0.4 N NaOH, and treated twice with 1 M Tris-HCl (pH 7.5). Dried membranes were probed with a ^{32}P-labeled probe encompassing the repeated viral *c* sequence, as described previously (57).

Indirect IF. Cells were grown on coverslips, adsorbed for 1 h with 10 to 20 PFU of the desired virus per cell, and, unless otherwise indicated, incubated for 5.5 h postadsorption in the presence or absence of ACV (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 PBS (pH 7.4) for 30 min, and permeabilized with 1.0% Triton X-100 for 10 min. If BrdU was incorporated, cells were treated with 4 M HCl for 10 min to expose the BrdU. UL29 was detected with 3-83 diluted 1:500 in 3% normal goat serum (NGS) in PBS for 30 min or with α-ICP8 diluted 1:200 in 3% NGS in PBS for 30 min. Incorporated BrdU was detected with the monoclonal antiserum diluted 1:200 in 3% NGS in PBS for 30 min. The PML protein was detected with the PML antibody diluted 1:200 in 3% NGS in PBS for 30 min. Cells were reacted with fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse antibodies diluted 1:200 in 3% NGS in PBS for 30 min at room temperature. All double-labeled cells were checked for bleed-through staining and cross-reactivity by treatment with only a single antibody and both secondary conjugates. No evidence for artifactual staining patterns was observed. Coverslips were mounted in glycerol gelatin containing 2.5% DABCO to retard bleaching.

Imaging. Cells were imaged on a Zeiss Axiovert 135 laser scanning microscope (confocal) equipped with a Zeiss X63 Plan Neofluar objective. Collected images were arranged and labeled with a Silicon Graphics workstation equipped with Adobe Photoshop 3.0.

RESULTS

We proposed previously that the lack of or inhibition of an active viral polymerase may result in the appearance of single-stranded DNA on which UL29 can associate, leading to the appearance of prereplicative sites (26). We speculated that if specific inhibition of the viral polymerase is necessary and sufficient for prereplicative site formation, a double mutant lacking an essential replication gene as well as UL30 should form prereplicative sites in the absence of polymerase inhibitors (26). In this report, a chimera of the UL5 null mutant *hr*99 (57) and the UL30 null mutant HP66 (31) was constructed to test this hypothesis. Furthermore, a chimera of *hr*94 (UL9 null mutant) and *hr*99R345K, a replication-defective UL5 helicase

motif mutant virus, was designed to test the idea that either of the HSV-1 DNA helicases, UL5 in the helicase-primase complex or the origin-binding protein UL9, is sufficient for the formation of prereplicative sites. In order to propagate these double mutant viruses, permissive cell lines containing two replication genes were constructed.

Isolation of permissive cell lines for double mutants. Cell lines permissive for the propagation of mutants lacking polymerase and UL5 were isolated by stable cotransfection of a construct expressing UL5 and a selectable hygromycin resistance marker into the B3 cell line, which expresses UL30 and is G418 resistant. Doubly resistant cell clones were isolated as described in Materials and Methods. The screening process for complementing cell lines was based on the observation that replication compartments in infected cells only form in the presence of all seven essential viral replication proteins. Thus, G418/hygromycin-resistant colonies were screened for the ability to complement *hr*99 and HP66 for replication compartment formation as detected by IF of UL29 localization. The use of IF to screen potential complementing cell lines proved to be very rapid and more efficient than conventional methods of assaying for the ability of each single mutant to form plaques on each candidate cell line. Once a double cell line was identified by IF, plaque formation by each virus was assessed. The cell line which supported the largest, most efficient plaque formation for both HP66 and *hr*99, DTMA, was chosen to propagate a UL5/UL30 double mutant as described in the next section.

A cell line expressing UL5 and UL9 was generated by stable cotransfection of a construct expressing UL5 and a selectable hygromycin resistance marker into the 2B11 cell line, which expresses UL9 and is G418 resistant. Cell lines were screened as described above, except that *hr*94 (UL9 null mutant) and *hr*99 (UL5 null mutant) were used to test for complementation by IF. The cell line which supported the most efficient plaque formation for both *hr*99 and *hr*94, Hel28, was chosen to propagate a UL5/UL9 double mutant as described below.

Construction of the UL5/UL30 double mutant 66/99 and the UL5/UL9 double mutant RK/94. While analyzing the ability of the DTMA cell line to complement HP66 and *hr*99, we found that HP66 forms light blue plaques, whereas *hr*99 forms dark blue plaques. To isolate the UL5/UL30 double mutant virus, DTMA cells were coinfected with HP66 and *hr*99 at a 10:1 ratio. The ratio was chosen so that a dark blue plaque (*hr*99 phenotype) would be more likely to also contain an inactivated UL30 gene (light phenotype masked by the *hr*99 phenotype). Such dark blue plaques were picked, and titers of aliquots were determined in parallel on singly complementing cell lines L2-5 and B3 and the doubly complementing cell line DTMA. Plaques that grew only on DTMA cells were picked for another round of plaque purification as described above. One plaque out of 20 analyzed formed plaques on DTMA cells and not on L2-5 or B3 cells. This virus was designated 66/99; a viral stock was propagated on DTMA cells, and the titer on DTMA, L2-5, and B3 cells was determined. The titer was 1.6×10^7 PFU/ml on DTMA; however, on L2-5 and B3, the titer was $<10^3$ PFU/ml.

We initially attempted to make a UL5/UL9 double mutant as a chimera of *hr*99 and *hr*94, each of which contains an ICP6::*lacZ* insertion in the UL5 and UL9 genes, respectively (28, 57). However, the UL5/UL9 double mutant could not be obtained. We therefore decided to create a chimera of a replication-defective UL5 helicase motif mutant virus, R345K, and *hr*94 by infecting Hel28 cells at a ratio of 10:1. R345K forms white plaques, and *hr*94 forms blue plaques (28, 57), and we reasoned that some fraction of blue plaques picked under these conditions would be the desired double mutant. Four independent plaques out of 54 plaques gave rise to viral stocks that could grow on Hel28 cells but not on cell lines expressing only UL9 (2B11) or UL5 (L2-5). One candidate double mutant, designated RK/94, was amplified, and a viral stock was propagated on Hel28 cells. The titer of this stock was 6×10^7 PFU/ml on Hel28; however, this virus was unable to form plaques on 2B11 or L2-5 cells (titers of $<10^3$ PFU/ml). Therefore, RK/94 behaves as expected for a UL5/UL9-defective chimera.

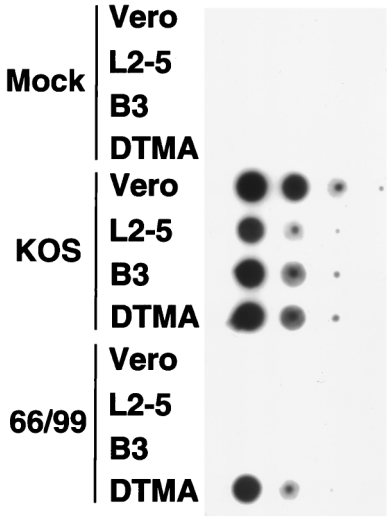

FIG. 1. DNA synthesis by 66/99. Vero and complementing cell lines were infected with the indicated viruses at an MOI of 10 PFU/cell and incubated for 20 h. Suspensions of cells were dot blotted onto a membrane in fivefold dilutions as described in Materials and Methods. The membrane was probed with a radiolabeled fragment from within the repeated viral *c* sequence.

Southern blot analysis of double mutant DNA. To confirm that 66/99 contained disrupted UL5 and UL30 genes and that RK/94 contained the desired mutations in the UL5 and UL9 genes, Southern blot analysis was performed with HSV- and *lacZ*-specific DNA probes (25). DNA from 66/99 contains both parental insertions, and DNA from RK/94 contains both the *Eco*RI site polymorphism in R345K and the expected *lacZ* insertion in *hr*94, confirming that both double mutants contain the expected defective parental alleles (25).

Analysis of protein production by double mutant viruses. To confirm that 66/99 contains the null alleles for UL5 and UL30, samples of infected Vero and DTMA cells were subjected to immunoblotting with antibodies recognizing UL5 or UL30. The results of this analysis confirm not only that the DTMA cell line expresses UL30, but also that Vero cells infected with 66/99 do not produce detectable UL5 or UL30 (25). The single UL5 mutant containing a point mutation, *hr*99R345K, was previously shown to produce a detectable but variant version of the UL5 protein; thus, Vero cells infected with the RK/94 double mutant would be expected to contain UL5 but not UL9 protein. This result was confirmed by immunoblot analysis with antibodies recognizing UL5 or UL9 (25).

Double mutants synthesize DNA only on cell lines containing both viral genes. Viral DNA synthesis was measured by dot blot hybridization of total DNA from infected cells with a virus-specific probe. As shown in Fig. 1, 66/99 synthesizes DNA on the DTMA cell line but not on Vero, L2-5, or B3 cells. In contrast, KOS synthesizes viral DNA on all of these cell lines. Furthermore, the probe did not cross-hybridize with mock-

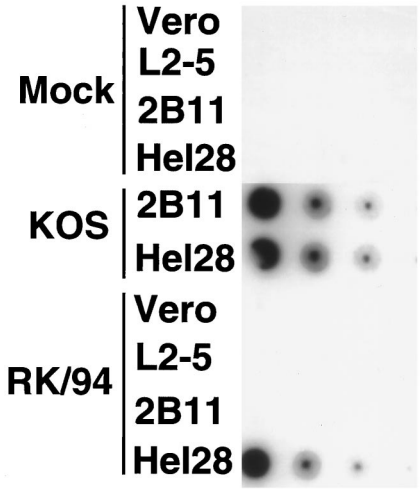

FIG. 2. DNA synthesis by RK/94. Vero and complementing cell lines were infected with the indicated viruses as described in the legend to Fig. 1. Suspensions of cells were dot blotted onto a membrane in fivefold dilutions as described in Materials and Methods. The membrane was probed with a radiolabeled fragment from within the repeated viral *c* sequence.

infected Vero, B3, L2-5, or DTMA cells. Although some variability in the ability of the various cell lines to support viral DNA synthesis was observed, it is clear that 66/99 is capable of DNA synthesis only when grown on the DTMA cell line. The same probe was used to show that RK/94 synthesizes DNA on the Hel28 cell line but not on Vero, L2-5, or 2B11 cells (Fig. 2). In contrast, KOS synthesizes DNA on 2B11 and Hel28 cells; once again the probe did not cross-hybridize with any of the four cell lines. These results confirm that 66/99 and RK/94 are only able to synthesize viral DNA when both mutations are complemented by DTMA or Hel28 cells, respectively.

Confirmation of the identity of the double mutants by UL29 localization. We and others have established that replication-defective null and point mutant viruses exhibit characteristic IF staining patterns for UL29 in infected cells (summarized in Table 1) (24, 26). Vero cells infected with the UL30 mutant HP66 contain UL29 in prereplicative sites in the presence and absence of polymerase inhibitors (2) (Table 1). UL5 mutant (*hr*99)-infected Vero cells contain UL29 in a diffuse pattern that can be converted to a prereplicative site pattern by the addition of polymerase inhibitors (24, 26) (Table 1). We compared the UL29 localization patterns in cells infected with the double mutant viruses to the patterns seen with single mutants to further confirm their identity. L2-5, B3, and DTMA cells were infected with the UL5/UL30 double mutant 66/99 in the presence or absence of the polymerase inhibitors PAA and ACV. As expected, cells infected with wild-type virus (KOS) contained replication compartments in the absence of drugs (Fig. 3A), but exhibited the prereplicative type pattern when treated with PAA and ACV (Fig. 3B and C, respectively). As shown in Fig. 3D to F, B3 cells infected with 66/99 exhibited a diffuse UL29 staining pattern that could be converted to a prereplicative site pattern with PAA or ACV (summarized in Table 2). This is the expected phenotype, since the B3 line expresses polymerase but UL5 is still lacking in the infection. L2-5 cells infected with 66/99 contained prereplicative sites in the presence and absence of PAA, demonstrating that expression of UL5 in *trans* converts the 66/99 phenotype to that of HP66 (Fig. 3G to I). DTMA cells infected with 66/99 contained UL29 in replication compartments and in prereplicative sites when treated with PAA or ACV (Fig. 3J to L). These results confirm that the defects in 66/99 can be complemented by expression of UL5 and UL30 and thus verify its identity as a UL5/UL30 double mutant.

TABLE 1. UL29 staining pattern in wild-type and mutant-infected Vero cells in the presence and absence of PAA[a]

Virus (mutant gene)	Result with[b]:	
	No drug	PAA
KOS (none)	RC	PR
*hr*99 (UL5)	D	PR
*hr*99R345K (UL5)	D	PR
*hr*94 (UL9)	D	PR
HP66 (UL30)	PR	PR

[a] Data were previously reported (26).
[b] RC, UL29 staining in replication compartments; PR, UL29 staining in prereplicative sites; D, diffuse UL29 staining.

Confirmation of the identity of RK/94 was carried out by infecting L2-5, 2B11, and Hel28 cells with RK/94 in the presence or absence of PAA or ACV. The IF staining pattern for UL29 in these infected cells was then examined and compared to that of the parental viruses R345K and *hr*94. Vero cells infected with R345K (UL5 mutant) or *hr*94 (UL9 mutant) contain UL29 in a diffuse pattern that can be converted to a prereplicative site pattern by the addition of polymerase inhibitors (24, 26) (Table 1). As shown in Fig. 4A to C, L2-5 cells infected with RK/94 contained UL29 in a diffuse staining pattern that could be converted to a prereplicative site pattern with PAA or ACV (summarized in Table 2). This is the expected phenotype, since RK/94 infection of L2-5 cells would resemble an *hr*94 infection of Vero cells (i.e., only UL9 would be lacking). 2B11 cells infected with RK/94 contained UL29 in a diffuse staining pattern that could be converted to a prereplicative site pattern with PAA or ACV (Fig. 4D to F). This too is expected because this infection would resemble Vero cells infected with R345K (i.e., no functional UL5 expression). Hel28 cells infected with RK/94 contained UL29 in replication compartments (Fig. 4G) and in prereplicative sites when treated with PAA and ACV (Fig. 4H and I, respectively). These results confirm that the defects in RK/94 can be complemented by expression of UL5 and UL9 and thus verify its identity as UL5/UL9 double mutant.

UL29 localization in Vero cells infected with double mutant viruses. The UL5/UL30 double mutant 66/99 was constructed to test the hypothesis that it is simply the lack of an active polymerase that is needed for prereplicative site formation and that PAA and ACV facilitate prereplicative site formation in UL5-defective mutant-infected Vero cells by specifically inactivating UL30 (26). We predicted that the double mutant lacking UL5 and UL30 would form prereplicative sites in the absence of polymerase inhibitors. Vero cells were infected with 66/99 in the presence or absence of PAA or ACV. As shown in Fig. 3M, UL29 was present in a diffuse nuclear pattern in 66/99-infected Vero cells, a finding not consistent with our prediction that prereplicative site formation in *hr*99-infected cells is due to specific inactivation or lack of UL30, since the 66/99-infected cells do not contain UL30. In the presence of PAA, 66/99-infected Vero cells contained UL29 in a diffuse, nuclear pattern (Fig. 3N). This result shows that PAA itself is not sufficient to cause prereplicative site formation, as previously suggested by Liptak et al. (24). However, UL29 was localized to prereplicative sites in cells treated with ACV (Fig. 3O). The experimental results do not completely support our

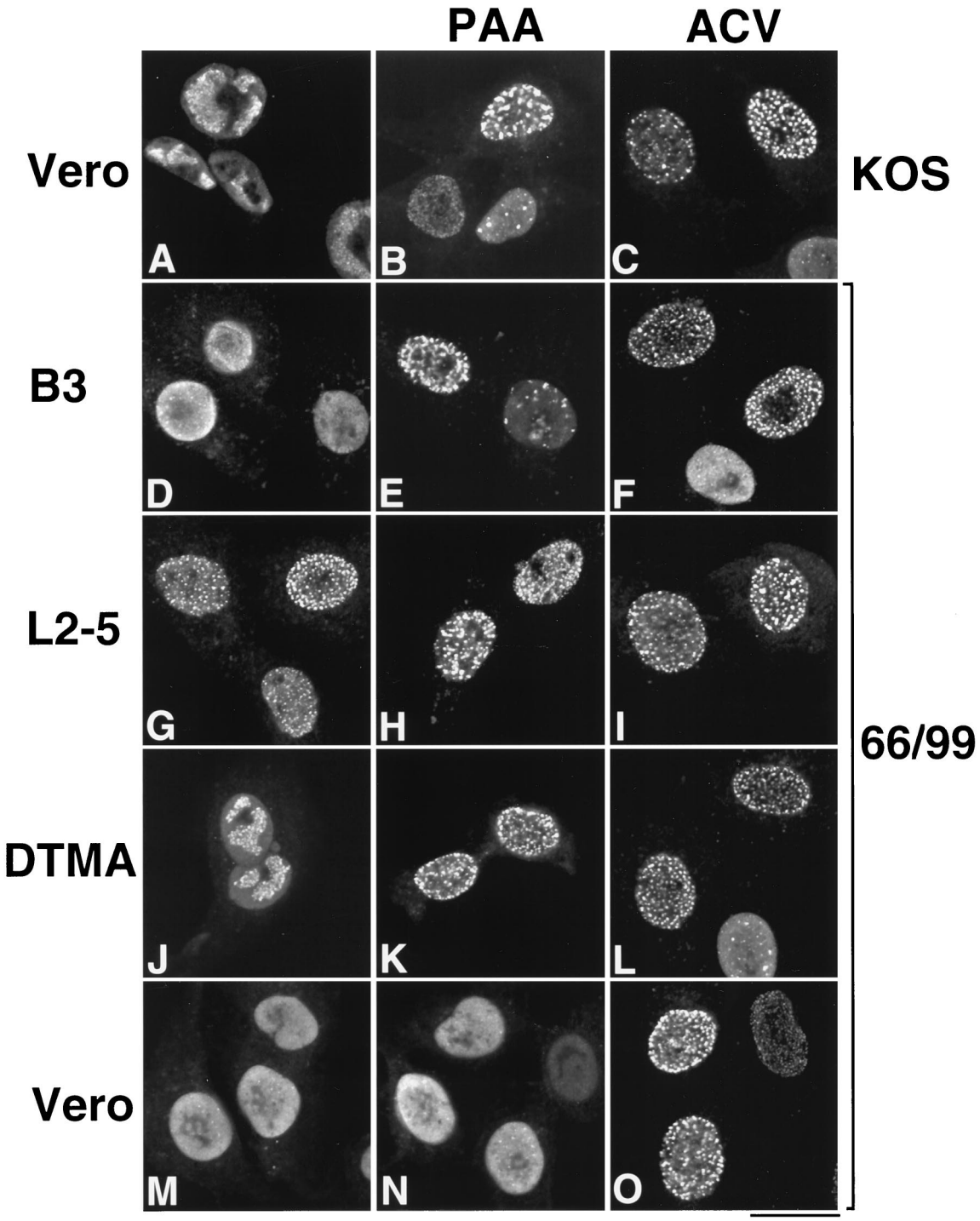

FIG. 3. Localization of UL29 in cells infected with 66/99. Vero and complementing cells as indicated were infected at an MOI of 10 to 20 PFU/cell, incubated for 5.5 h in the presence or absence of PAA or ACV, and stained with 3-83 according to the procedures described in Materials and Methods. (A to C) Vero cells infected with KOS. (D to F) B3 cells (containing the HSV-1 UL30 gene) infected with the UL5/UL30 double mutant 66/99. (G to I) L2-5 cells (containing the HSV-1 UL5 gene) infected with 66/99. (J to L) DTMA cells (containing both the UL5 and UL30 genes) infected with 66/99. (M to O) Vero cells infected with 66/99. Bar, 15 μm.

previous model; alternative models will be considered in the Discussion.

The UL5/UL9 double mutant RK/94 was constructed to test the hypothesis that either of the HSV-1 proteins that exhibit helicase activity, UL5 in the helicase-primase complex or UL9, is sufficient for the formation of prereplicative sites. Vero cells were infected with RK/94 in the presence or absence of PAA or ACV. As shown in Fig. 4J, UL29 was present in a diffuse nuclear pattern in RK/94-infected Vero cells. The UL29 staining pattern was, however, converted to a prereplicative pattern by the addition of PAA or ACV (Fig. 4K and L, respectively). Thus, as long as polymerase inhibitors are present, neither

TABLE 2. UL29 staining in cells infected with double mutants in the presence and absence of polymerase inhibitors

Cell line (viral gene)	Result with[a]:		
	No drug	PAA	ACV
66/99[b] infection			
B3 (UL30)	D	PR	PR
L2-5 (UL5)	PR	PR	PR
DTMA (UL5 + UL30)	RC	PR	PR
Vero (none)	D	D	PR
RK/94[c] infection			
L2-5 (UL5)	D	PR	PR
2B11 (UL9)	D	PR	PR
Hel28 (UL5 + UL9)	RC	PR	PR
Vero (none)	D	PR	PR

[a] D, diffuse UL29 staining; PR, UL29 staining in prereplicative sites; RC, UL29 staining in replication compartments.
[b] UL5/UL30 double mutant.
[c] UL5/UL9 double mutant.

UL9 nor functional UL5 is necessary for the prereplicative site pattern to form. The implications of these results will be discussed below.

Prereplicative site formation in cells infected with KOS in the presence of PAA. The finding that the behavior of the double mutants described in this report did not support our previous model led us to reexamine the prereplicative site pattern formed in cells infected with wild-type virus in the presence of PAA. We were particularly interested in whether any of these sites could be considered intermediates in the formation of replication compartments. Because of the recent finding that input viral DNA is deposited in close proximity to ND10 (18, 35) and that replication compartments form at the periphery of ND10 (18, 35), we were interested in the relationship of the prereplicative site pattern to ND10. HEp2 cells were used for these experiments because the PML antibody used as a marker for ND10 does not react with Vero cell ND10. Since HSV-1 infection is known to disrupt ND10 (34), cells were infected for only 4 h; at this time, ND10 disruption is not complete and these sites can still be visualized, although they are somewhat less well defined than ND10 seen in uninfected cells. HEp2 and Vero cells infected with wild-type virus in the presence of PAA were examined by confocal microscopy and double IF labeling. Two distinct staining patterns were evident. Some UL29-positive cells contain numerous (greater than 20) punctate foci which represent the pattern most often depicted in the literature as prereplicative sites (Fig. 5A and G) (2, 6, 15, 24, 26, 42). Although cells with this pattern are often photographed in a focal plane such that foci appear distributed throughout the nucleus (upper cell in Fig. 5A), confocal microscopic sectioning in more central focal planes reveals that these foci tend to localize predominantly at the periphery of the nucleus (lower cell in Fig. 5A). A second subset of cells was identified which contain fewer UL29 foci (less than 15), and these foci tend to be localized towards the interior of the nucleus (Fig. 5D and J). To determine whether either of these patterns was associated with ND10 domains, 100 cells which had been double stained for UL29 and PML were counted and classified according to their UL29 staining pattern. We observed colocalization of UL29 with PML in 91% of the cells classified as having few foci (Fig. 5D to F) but in only 11% of the cells classified as having numerous foci (Fig. 5A to C). In those cells with numerous foci in which colocalization was observed, only a few of the individual foci colocalized with PML (less than five foci per cell). Three criteria can thus be used to distinguish the two staining patterns: numerous foci as opposed to few, peripheral as opposed to centrally located, and lack of PML association as opposed to association with PML (ND10). We designated the second pattern consisting of fewer (less than 15 foci) centrally distributed UL29 foci which colocalize predominantly with PML as the ND10-associated pattern.

Previous descriptions of the prereplicative site pattern suggested that UL29 localizes to numerous punctate foci, many of which also colabel with BrdU (6, 42). Therefore, we examined the "numerous" and ND10-associated staining patterns for BrdU labeling and UL29 staining in KOS infected-Vero cells in the presence of PAA. Vero cells were used in this experiment because most of the previous work was carried out with these cells; however, similar results were obtained in KOS-infected HEp2 cells (1a). One hundred cells were classified as either containing numerous or ND10-associated UL29 sites. We observed colocalization of BrdU with UL29 in 97% of the cells which contain numerous foci (Fig. 5G to I); conversely, colocalization was observed in only 27% of the cells with ND10-associated foci (Fig. 5J to L). A majority of the 27% of cells with ND10-associated foci which colabel with BrdU did not show extensive BrdU incorporation throughout the nucleus; instead, they contained unusually large PML foci with faint BrdU staining that is reminiscent of very early replication compartments (1a). In summary, the majority of the numerous sites colabel with BrdU, while the majority of the ND10-associated sites do not. Since viral DNA synthesis is inhibited by PAA, the cells exhibiting numerous UL29 foci and BrdU staining must be in S phase or in the process of DNA repair synthesis. UL29, a single-strand-binding protein, may accumulate at cellular replication forks to form the numerous UL29 foci (see Discussion).

The association between ND10 and the process of active viral DNA replication is further supported by our observation that in cells infected with wild-type virus in the absence of polymerase inhibitors, PML staining can be observed within replication compartments (Fig. 6). We previously showed that another ND10 antigen, a 55-kDa protein detected by monoclonal antibody MAb138, is recruited into replication compartments formed in cells transfected with the seven replication genes (27). The observations that at least two ND10 antigens are recruited into replication compartments and that replication compartments are formed in close proximity to ND10 suggest that the ND10-associated structures seen in this report are likely to represent sites at which replication compartments are destined to form.

DISCUSSION

Considerable confusion has arisen over the pattern of UL29 staining in HSV-1-infected cells in cells which have been treated with viral polymerase inhibitors (2, 6, 15, 24, 26). The typical prereplicative site pattern consists of numerous UL29 foci, many of which colabel with BrdU. Since this pattern can form in cells infected with mutants which are defective in UL5, UL8, UL9, or UL52 in the presence of polymerase inhibitors (24, 26), we proposed that it is unlikely that these numerous UL29 foci actually represent a functional subassembly of viral replication proteins that could lead to the formation of replication compartments (26). On the other hand, de Bruyn Kops and Knipe observed that permissive cells infected with wild-type virus contain UL29 structures at early times which resemble prereplicative sites and suggested that they may represent intermediates in the formation of replication compartments

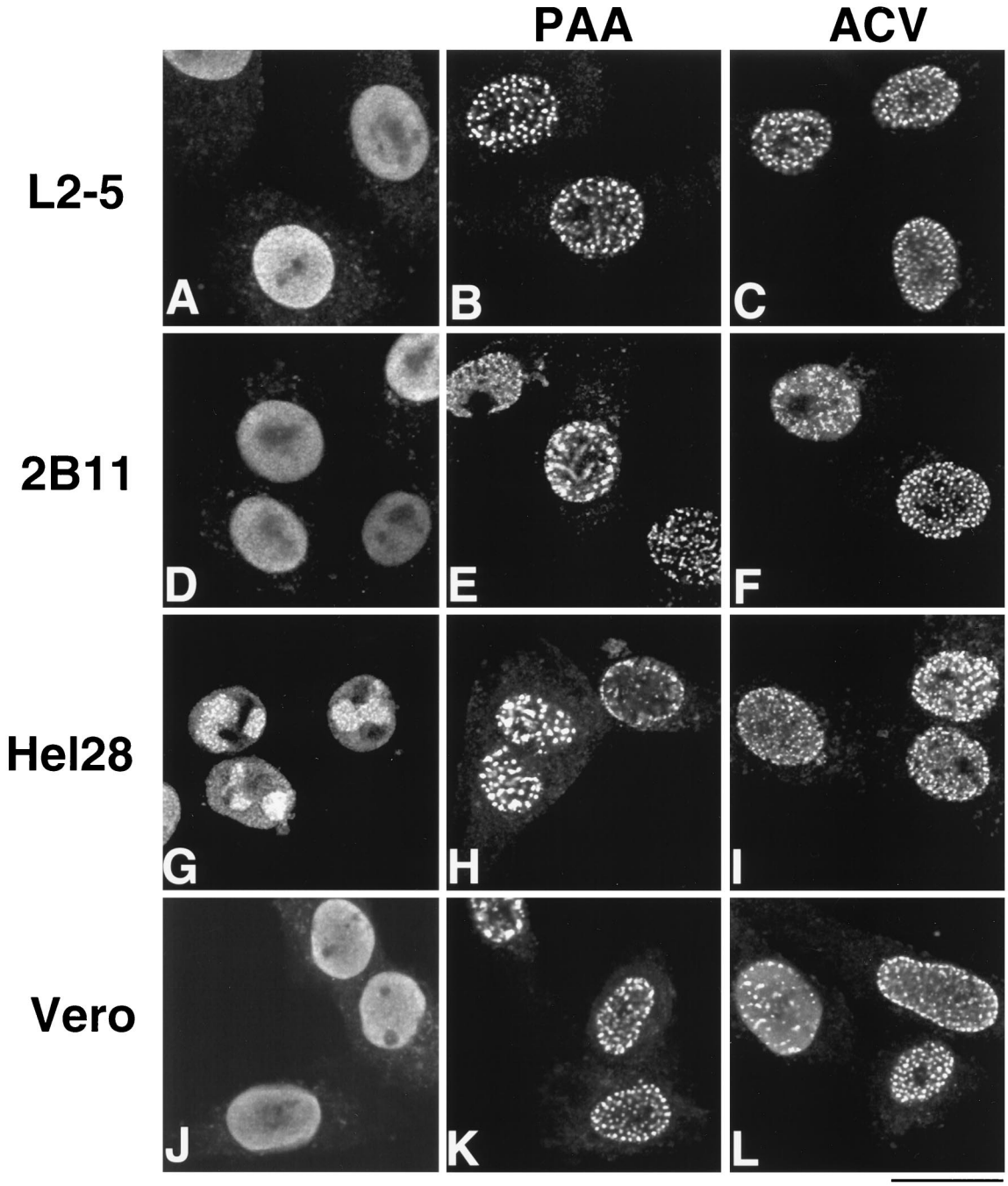

FIG. 4. Localization of UL29 in cells infected with RK/94. Vero and complementing cells were infected with the UL5/UL9 double mutant RK/94 and treated as described in the legend to Fig. 3. (A to C) L2-5 cells (containing the HSV-1 UL5 gene). (D to F) 2B11 (containing the HSV-1 UL9 gene). (G to I) Hel28 cells (containing both UL5 and UL9). (J to L) Vero cells. Bar, 15 μm.

(6). This observation made in the absence of polymerase inhibitors led us to consider the possibility that heterogeneity may exist in the typical prereplicative site pattern. We have taken a closer look at cells infected with wild-type virus in the presence of polymerase inhibitors. In cells infected with wild-type HSV-1 in the presence of polymerase inhibitors, at least two different UL29 staining patterns can be detected. In some cells, numerous (generally more than 20) punctate foci are detected, many of which colocalize with BrdU. In other cells, fewer punctate foci were detected, and these structures do not colocalize with BrdU; however, they colocalize with PML-containing ND10 domains. Thus, the previously described prereplicative site pattern is most likely composed of two or more localization patterns which are not functionally equivalent.

Several lines of evidence support the model that the ND10-associated structures are destined to become sites of viral replication. (i) Viral DNA is deposited at the periphery of ND10 (18). (ii) Replication compartments form in close association with ND10 both in infected cells and in cells transfected with the seven HSV-1 replication proteins (27, 35). (iii) Some ND10

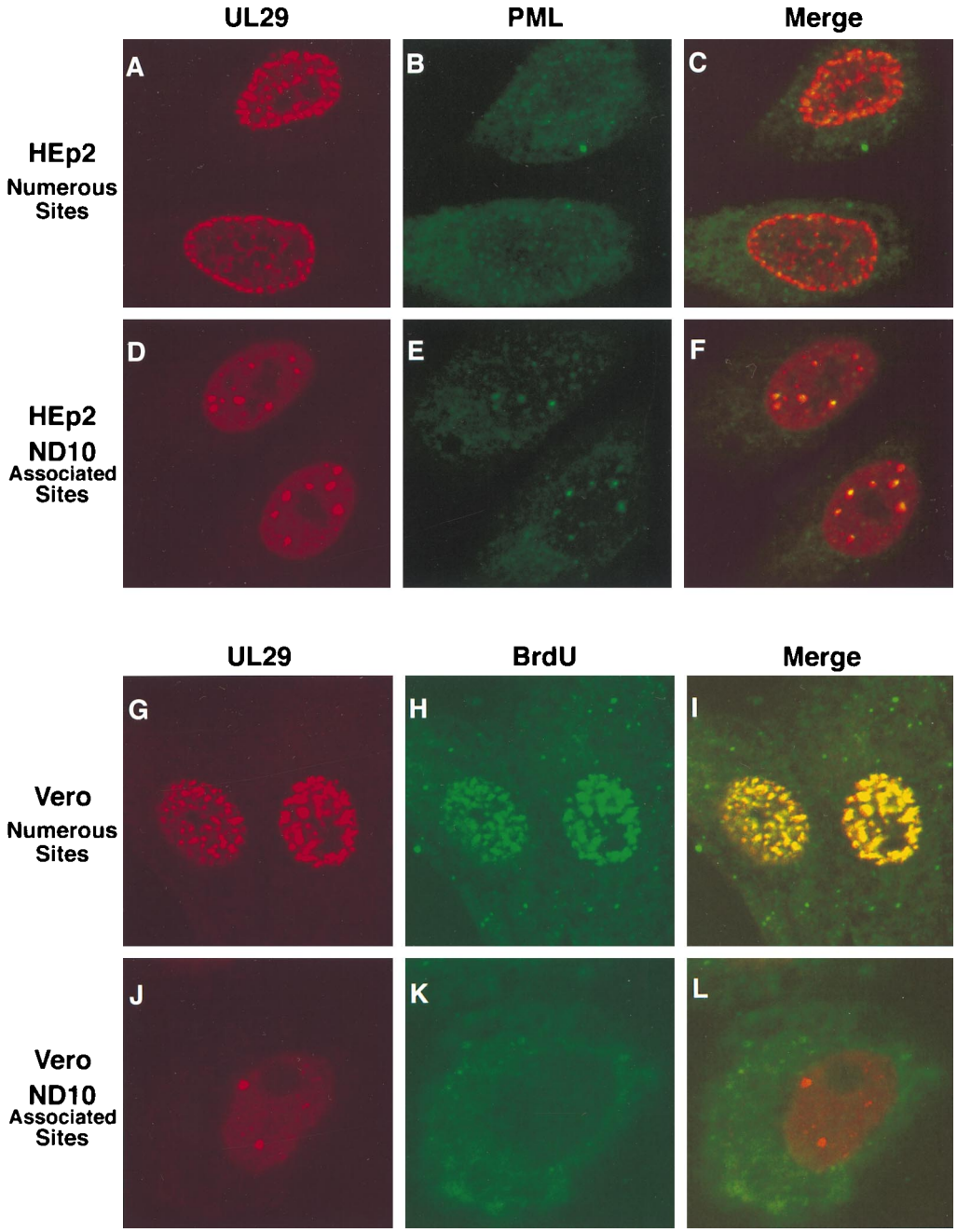

FIG. 5. Heterogeneity of prereplicative sites in KOS-infected HEp2 and Vero cells in the presence of PAA. HEp2 (A to F) and Vero (G to L) cells were infected with KOS at an MOI of 10 to 20 PFU/cell for 4 h in the presence of PAA. (A, D, G, and J) Staining with a polyclonal antibody (α-ICP8) obtained from Bill Ruyechan. (B and E) Staining with anti-PML antibody. (C and F) Merged image. (H and K) Staining with anti-BrdU antibody. (I and L) Merged image.

antigens are recruited into replication compartments formed either by infection (this work) or by transfection (27). This model is also consistent with the previous finding that the number of replication compartments in infected cells does not increase appreciably beyond a dozen, even at high MOIs (18, 35), which parallels the number of ND10 per cell.

Further characterization of the numerous UL29 staining pattern. In this paper, we have further tested the requirements for the numerous UL29 staining pattern by using double mutant viruses defective in more than one replication protein. We suggest that the numerous pattern of UL29 staining is caused

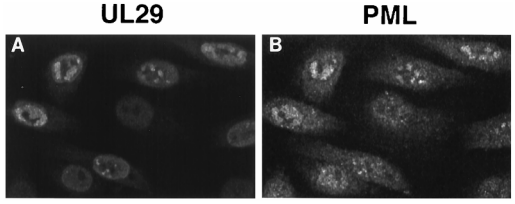

FIG. 6. Colocalization of PML and UL29 in replication compartments. HEp2 cells were infected with KOS at an MOI of 10 to 20 PFU/cell for 5 h in the absence of PAA. (A) Staining with α-ICP8. (B) Staining with anti-PML.

by the accumulation of UL29 and other replication proteins at regions of single-stranded cellular DNA exposed as a result of a perturbation at a cellular replication fork. Such a perturbation might be caused by uncoupling of elongation and unwinding. Fork uncoupling has been associated with UV light-induced DNA damage and inhibition of cellular replication proteins such as polymerases and topoisomerases and can lead to formation of gaps, DNA breaks, and eventually to genetic instability (reviewed in reference 49). According to this model, inhibition of polymerase activity in infected cells can uncouple helicase machinery from DNA elongation and expose stretches of single-stranded DNA at which UL29 can accumulate. Uncoupling of replication forks may be caused by inhibition of viral and/or cellular polymerases as long as viral or cellular helicases are still active. This proposal is consistent with the observation that both viral replication machinery and cellular replication machinery are present in the numerous prereplicative sites (2, 10, 15, 24, 26, 55).

According to this scenario, in wild-type HSV-1-infected cells in the presence of polymerase inhibitors, the viral polymerase is inactivated; however, the helicase would be expected to continue unwinding DNA (49). This may lead to the appearance of single-stranded DNA and the accumulation of UL29 at numerous sites wherever DNA is being replicated. A similar uncoupling would occur in cells infected with polymerase mutants without the need for polymerase inhibitors, consistent with previous observations (2, 43). In a cell infected with mutants defective in helicase-primase or UL9, numerous sites form only in the presence of polymerase inhibitors (24, 26). A similar result was obtained in cells infected with a UL5/UL9 double mutant (this paper). In these situations, inhibition of the viral polymerase may still cause uncoupling at the replication fork, since the cellular helicase machinery would be expected to remain functional. These results imply that the viral polymerase protein is present at the replication fork. In the absence of polymerase inhibitors, UL29 staining is diffuse, presumably because no uncoupling occurs. In this situation, synthesis of DNA by viral proteins does not occur because of the lack of required replication proteins. Taken together, these results indicate that the numerous UL29 staining pattern can occur in the absence of several replication proteins. In fact, the only viral protein absolutely essential for the formation of this type of pattern is UL29 (6); other viral replication proteins, UL5, UL8, UL9, UL30, UL42, and UL52, appear to be dispensable for this pattern to form as long as agents leading to uncoupling of the replication fork are present (24, 26). This lack of requirement of individual HSV-1 replication proteins for the formation of this numerous UL29 staining pattern indicate that they are unlikely to represent intermediates in the formation of functional replication compartments.

We previously predicted that in a cell infected with a mutant defective in both UL5 and UL30, prereplicative sites would form even in the absence of polymerase inhibitors (26); however, diffuse UL29 staining was observed. In this situation, the viral helicase and polymerase are not even present at the replication fork, and the cellular replication machinery at the fork should not be affected. Under these conditions, no uncoupling would occur and the UL29 pattern would be diffuse. Surprisingly, although cells infected with the UL5/UL30 double mutant exhibited diffuse UL29 staining in cells treated with PAA, they exhibit a numerous type UL29 pattern in cells treated with ACV. The ability of ACV but not PAA to facilitate prereplicative site formation in the UL5/UL30 double mutant-infected Vero cells may reflect the different mechanisms by which these drugs inhibit polymerases (reviewed in reference 5). PAA acts noncompetitively by mimicking pyrophosphate (23, 30). ACV

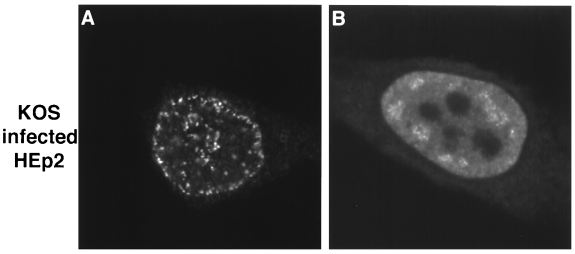

FIG. 7. UL29 staining in KOS infected-HEp2 cells with and without treatment with UV light. HEp2 cells were infected with KOS at an MOI of 20 PFU/cell for 4 h. (A) Cells UV treated just after the absorption period at a distance of 75 cm with a Sylvania 30-W bulb in a sterileGARD Hood for 30 s. (B) Control cells infected as in panel A but not treated with UV light.

is phosphorylated to acyclo-GMP by HSV-1 thymidine kinase and then to its active species, acyclo-GTP, by cellular kinases (11, 14, 37, 38); following activation, acyclo-GTP acts to inhibit UL30 both by competitively inhibiting binding of dGTP to polymerases (11, 13) and by incorporation into elongating DNA (13), where it acts as a chain terminator (36, 44, 45). The ability of ACV-triphosphate (ACV-TP) but not PAA to be incorporated into elongating DNA may account for the ability of ACV to facilitate prereplicative site formation in the UL5/UL30 double mutant, since the incorporation of a chain terminator may lead to DNA damage. According to this scenario, ACV may cause damage even when the viral polymerase is not present. The presence of viral thymidine kinase in cells infected with the UL5/UL30 double mutant will result in the generation of ACV-TP, and although the apparent K_i values for cellular polymerases are orders of magnitude higher than that for HSV polymerase, ACV-TP has also been demonstrated to inhibit cellular polymerases (Pol δ, Pol ε, and Pol α associated with its primase subunit) (17, 32). Thus, at the concentrations of ACV used in this study, it is possible that incorporation of the chain terminator at cellular replication forks may result in DNA damage and uncoupling leading to the accumulation of UL29 at numerous foci. PAA cannot be incorporated and thus would not be expected to cause this type of damage.

The uncoupling model predicts that the prereplicative site pattern would form under other conditions which would cause uncoupling at a replication fork. Fork uncoupling caused by UV light is known to cause gap formation in cellular DNA (51). To test this prediction of the model, Vero cells infected with wild-type virus were irradiated with UV light as described in the legend to Fig. 7; this treatment resulted in the numerous pattern of UL29 staining (Fig. 7). In this case, direct DNA damage may lead to the generation of single-strand DNA or DNA breaks which could cause the accumulation of UL29. Further experiments will be needed to establish the relationship between polymerase inhibition, DNA damage, and sites of UL29 accumulation.

In summary, we have found that the previously described prereplicative site staining pattern of UL29 is composed of a mixture of at least two staining patterns. Some cells contain numerous (more than 20) UL29 foci which colabel with BrdU and may indicate accumulation of UL29 at an uncoupled cellular replication fork. The other population of cells in the prereplicative pattern contains fewer sites of UL29 staining which are ND10 associated. It is possible that cells which contain numerous UL29 sites also contain some ND10-associated sites which are masked by the large numbers of the numerous sites. It is notable that other DNA viruses like simian virus 40

and adenovirus also deposit their viral DNA next to ND10 and establish replication compartments in close association with these structures (4, 8, 18). It will be of interest to determine the role of ND10 in the initiation of the transcription and replication programs of HSV-1 in the host cell.

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