Assembly of Herpes Simplex Virus Replication Proteins at Two Distinct Intranuclear Sites

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Herpes simplex virus DNA replication proteins amplify the viral genome in large globular replication compartments within infected cell nuclei. In the absence of viral DNA synthesis, the replication proteins accumulate at punctate foci throughout the nucleus referred to as prereplicative sites. To more thoroughly understand the nature of this nuclear assembly process, we have examined the viral and cellular factors involved. First, we demonstrate that six viral replication proteins are sufficient for formation of functional replication compartments in transfected cells in the absence of viral origin-containing DNA. Second, we show that the viral replication proteins form two distinct types of prereplicative sites within infected cells. One type of punctate structure assembles in S-phase cells, colocalizes with cellular DNA synthesis, and contains components of the host-cell replication apparatus as indicated by the presence of Replication Protein A. However, the other class of prereplicative sites is independent of host-cell DNA synthesis as evidenced by their formation in cells arrested in G1 by n-butyrte. These complexes are significantly less abundant and closely correspond with cellular Nuclear Domain 10 structures to which viral DNA has recently been demonstrated to be targeted early in infection (G. G. Maul, A. M. Ishov, and R. D. Everett, 1996, Virology 217, 67-75). Hence, this second type appears to represent the subset of prereplicative sites destined to become replication compartments.

INTRODUCTION

Seven viral gene products are absolutely necessary for herpes simplex virus (HSV) DNA replication in infected tissue culture cells (reviewed in Challberg and Kelly, 1989; Knipe, 1989; Weller, 1991). Analysis of these proteins has defined them as a polymerase (UL30) and its processivity factor (UL42), a single-stranded DNA-binding protein (ICP8 or UL29), an origin-binding protein (UL9), and components of a helicase-primase complex (UL5, UL8, and UL52). In transient transfection assays, these seven genes are sufficient for amplification of a cotransfected plasmid containing a viral origin of replication (Challberg, 1986; Wu et al., 1988). In the absence of the origin-binding protein, the remaining six are also able to induce synthesis of non-HSV DNA (Heilbronn and Zur Hausen, 1989; Heilbronn et al., 1990).

HSV DNA replication takes place in large globular replication compartments within infected-cell nuclei (Rixon et al., 1983; de Bruyn Kops and Knipe, 1988). All seven of the essential replication proteins are present within these domains (Quinlan et al., 1984; de Bruyn Kops and Knipe, 1988; Olivo et al., 1989; Goodrich et al., 1990; Bush et al., 1991; Liptak et al., 1996; Lukonis and Weller, 1996). If viral DNA synthesis is blocked during infection with drugs that inhibit the HSV polymerase, a subset of the replication proteins accumulates in smaller structures with a punctate distribution throughout the nucleus which have been termed prereplicative sites (Quinlan et al., 1984; Olivo et al., 1989; Goodrich et al., 1990; Bush et al., 1991; Liptak et al., 1996; Lukonis and Weller, 1996). There is significant evidence to support the idea that at least some of these punctate structures are intermediates or precursors to the larger replication compartments (reviewed in Liptak et al., 1996). The colocalization of host cell DNA synthesis activity and related proteins, such as SS8 (or RF-A), PCNA, Rb, and p53, with prereplicative sites has previously suggested that these viral proteins become associated with sites related to cellular DNA synthesis (de Bruyn Kops and Knipe, 1988; Wilcock and Lane, 1991). However, input viral DNA appears to be initially targeted to fewer intranuclear sites at the peripheries of cellular Nuclear Domain 10 structures (ND10; Ishov and Maul, 1996; Maul et al., 1996).

Using seven mutant viruses, each deleted for one of the essential replication genes, we and others have recently shown that under natural infection conditions three components of the viral DNA replication machinery, the helicase-primase complex (UL5, UL8, and UL52), the origin-binding protein (UL9), and the single-stranded DNA-binding protein (ICP8), are all required for assembly of prereplicative sites within infected cells (Liptak et al., 1996; Lukonis and Weller, 1996). However, the addition of replication inhibitors could somehow compensate for the absence of UL5, UL8, UL52, or UL9 and allow punctate structures to form in helicase-primase and origin-binding protein mutant-infected cells, suggesting that the requirements for assembly of punctate sites can vary.
under different conditions. Indeed, although formation of prereplicative sites in wild-type (WT)-infected cells has been shown not to require cellular DNA synthesis (de Bruyn Kops and Knipe, 1988), in mutant-infected cells punctate structures formed only in the subset of cells in which active cellular DNA synthesis could be detected (Liptak et al., 1996). In addition, the requirements for viral protein assembly differed slightly in transfected cells in which ICP8 and the helicase–primase were sufficient for punctate structure formation in the absence of UL9 (Liptak et al., 1996). Hence, we found that the minimal viral and cellular factors necessary to promote assembly of viral replication protein complexes depended on the conditions being investigated.

To explain these differences in assembly requirements and identify the viral factors needed for replication compartment formation, we have continued to investigate the nature of viral replication structures. Extension of our previous transfection studies shows that even in the absence of viral origin-containing DNA, large globular replication compartments that synthesize DNA can be formed by a subset of the viral replication proteins. Further examination of the relationship between viral replication protein complexes and cellular DNA synthesis has indicated the existence of at least two distinct types of prereplicative sites. We demonstrate that these separate subclasses of punctate structures vary in their dependence on viral factors, host cell factors, and intranuclear location.

MATERIALS AND METHODS

Cells and viruses

All cell lines were grown and maintained in Dulbecco's modified Eagle's medium (Irvine Scientific, Santa Ana, CA) containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, streptomycin sulfate, and penicillin G potassium (DMEM - 10%FCS). Experiments were performed with Vero African green monkey kidney cells [American Tissue Culture Collection (ATCC), Rockville, MD] and WI38 human lung cells (ATCC) as indicated.

The HSV-1 WT strain KOS1.1 (Hughes and Munyon, 1975), originally received from M. Levine, was titered on Vero cells using an overlay of medium 199 (GIBCO, Gaithersburg, MD) containing 1% heat-inactivated calf serum and 0.1% human immune serum. The n212 ICP0 mutant virus containing a nonsense mutation in the ICP0 gene was obtained from Priscilla Schaffer (Cai and Schaffer, 1989).

HSV-1 WT strain KOS, derived from the same clinical isolate as KOS1.1, was originally obtained from Priscilla Schaffer. Replication protein mutant virus hr80 containing a lacZ insertion into the UL8 gene, as well as the complementing cell line, SL8, was provided by Sandra Weller (Carmichael and Weller, 1989). The UL42 deletion mutant virus, CgalΔ42, and the complementing U9 cell line were provided by Paul Johnson (Johnson et al., 1991). KOS was used as the WT strain in all experiments that used the replication protein mutants.

Reagents and antibodies

To arrest host cells in G1 phase without affecting viral replication, n-butyrate (Sigma Chemicals, St. Louis, MO) was added to the medium of the indicated cultures at a concentration of 3 mM for 6–12 hr prior to infection and in all solutions in which the cells were maintained during subsequent viral infections as previously described (Shadan et al., 1994). The viral-specific polymerase inhibitor sodium phosphonoacetate (PAA; Sigma) was included in the overlay media of infections at a concentration of 400 µg/ml when indicated, to prevent viral DNA replication (Leinbach et al., 1976; Mao et al., 1975).

Several different antibodies were used for ICP8 immunofluorescence staining as indicated. These included MAB LP-793 provided by Lenore Pereira, 10-E3 MAB received from Kathleen Shriver, 39S ascites fluid isolated from tumors formed by hybridoma cells obtained from ATCC (Showalter et al., 1981), and polyclonal antiserum 3-83 (Knipe et al., 1987). The anti-bromodeoxyuridine (anti-BrdU) monoclonal was purchased from Becton-Dickinson. MAB p70-9 recognizing the 70K subunit of cellular Replication Protein A (RP-A) was supplied by Bruce Stillman (Din et al., 1990). Human-specific anti-Sp100 antibody used to detect cellular ND10 sites was generously provided by Gerd Maul (Korieth et al., 1996).

Infections

Viruses were diluted in cold phosphate-buffered saline containing 0.1% glucose and 1% heat-inactivated newborn calf serum and absorbed to Vero cells at a multiplicity of infection (m.o.i.) of 20 PFU per cell. Alternatively, to establish a high-m.o.i. infection in WI38 cell cultures a Vero cell equivalent of 100 PFU per cell was absorbed. In both cases, after 1 hr at 37°C, the inoculum was removed and cells were overlaid with fresh medium. Standard infections were maintained in medium 199 containing 1% newborn calf serum. However, to more effectively determine the effects of n-butyrate-induced cell-cycle arrest, experiments using this reagent were modified so that infected cells were incubated in DMEM - 10%FCS to ensure that untreated control cultures were growing at optimal rates and hence would contain a higher percentage of S-phase cells. As described above, PAA was included in the overlay media of some infections to inhibit viral DNA synthesis.

Plasmids

pSV8.3 is a plasmid that expresses ICP8 using the SV40 early gene promoter (Gao et al., 1988; Gao and Knipe, 1992). The other six HSV replication proteins were expressed from the CMV IE promoter-driven constructs pCM-UL5, pCM-UL8, pCM-UL52, pCM-UL9, pCM-UL42,
and pCM-pol (Heilbronn and Zur Hausen, 1989) which were provided by Diane Hayward.

Transfections

Cells were grown on glass coverslips. At 70% confluency transfections were performed using calcium phosphate precipitation (Kingston, 1995). A total of 4 μg of DNA, consisting of 0.57 μg of each expression plasmid indicated plus pUC19 carrier DNA, was added to 1 ml DMEM – 10%FCS. The DNA precipitate was left on cells overnight (17–22 hr) at 37°C with 5% CO2 before changing to fresh DMEM – 10%FCS, and at 43 hr posttransfection cells were processed for immunofluorescence.

Indirect immunofluorescence

Cells were grown on glass coverslips as described above, and when indicated 3 mM n-butylate (Sigma) was added to this incubation medium to suppress host-cell DNA synthesis. At 6 hr postinfection (hpi) or 43 hr posttransfection, cells were fixed for 10 min in 2% formaldehyde and permeabilized for 2 min in –20°C acetone, and immunofluorescence was performed as previously described (Quinlan et al., 1984). Primary antibodies were used at the following dilutions: 395 (1:80), 3-83 (1:80), LP-793 (1:40), 10-E3 (1:40), p70-9 (1:40), anti-BrdU (1:10), and anti-Sp100 (undiluted). Fluorochrome-conjugated secondary antibodies used in all experiments included fluorescein-conjugated goat anti-rabbit IgG (1:200) and rhodamine-conjugated goat anti-mouse IgG (1:100) (Cappel Laboratories). Cells were mounted on glass slides in glycerol–gelatin (Sigma) containing 1.3 mg of p-phenyldiamine per milliliter and examined by fluorescence and phase-contrast microscopy with a Zeiss photomicroscope. Dual-image color micrographs were obtained by consecutive exposures with fluorescein and rhodamine filters (Knipe et al., 1987).

BrdU labeling

To allow detection of DNA synthesis, cells were labeled with the thymidine analog BrdU prior to fixation. Transfected cells were incubated with BrdU overnight (10–17 hr), while cells used in infection experiments and n-butylate assays were BrdU pulse-labeled for 30 min immediately before fixation. Immunofluorescence was performed as described above, except that after permeabilization, BrdU-labeled cells were treated with 4 M HCl to expose the incorporated BrdU residues.

RESULTS

Viral factors required for the assembly of replication compartments in transfected cells

To define the viral proteins necessary and sufficient for replication structure formation, we expressed various combinations of the seven essential viral replication genes in Vero cells by transient transfection. Immunofluorescence assays were performed at 43 hr posttransfection to monitor the localization of the transfected gene products. Because the association of ICP8 with discrete nuclear sites has been shown to be an essential early step in the assembly process within infected cells (de Bruyn Kops and Knipe, 1988), we chose ICP8 localization as the representative marker of HSV DNA replication structure formation. When ICP8 was introduced into cells alone, the protein entered the nucleus, but remained diffusely distributed in almost all cells (Fig. 1A). While some intrinsic ability to associate with nuclear substructure was detectable, less than 4% of ICP8-positive cells exhibited any specific intranuclear localization of ICP8 in the absence of other viral proteins. As observed previously, however, when the viral helicase–primase genes were coexpressed with ICP8, the staining pattern observed in 75% of the transfected cells was punctate, resembling that of infected-cell prereplicative sites (Fig. 1C) (Liptak et al., 1996). Expression of the viral processivity factor, UL42, did not noticeably alter the localization pattern of the ICP8/helicase–primase structures (Fig. 1E). Likewise, the presence of the catalytic UL30 polymerase subunit, in the absence of UL42, resulted in the same punctate ICP8 staining (results not shown). However, if the genes for both subunits of the HSV polymerase, UL30 and UL42, were included in the cotransfection with the ICP8 and helicase–primase genes, globular structures indistinguishable from infected-cell replication compartments formed (Fig. 1G). Although UL9 has been reported to have an inhibitory effect on the activity of the other HSV replication proteins in some situations (Perry et al., 1993; Stow et al., 1993; Skaliter and Lehman, 1994), the presence of the viral origin-binding protein did not significantly affect the localization of the other viral proteins to replication compartments (Fig. 1I). Hence, both components of the viral polymerase holoenzyme were required to induce this reorganization, but neither UL9 nor viral origin-containing DNA was needed.

To determine if these viral proteins were associated with and amplifying DNA at these assembly sites, DNA synthesis was assayed by immunofluorescence detection of BrdU incorporation. The distribution of ICP8 relative to sites of DNA synthesis was monitored by costaining with the 3-83 antiseraum. As expected, cells transfected with control pUC19 plasmid DNA expressed no ICP8 (Fig. 2A), but many exhibited granular BrdU staining throughout the nucleus indicative of active cellular DNA synthesis (Fig. 2B). When the six viral genes necessary for replication compartment formation were cotransfected (ICP8, UL5, UL8, UL52, UL30, and UL42), ICP8 staining revealed the presence of large globular inclusions (Fig. 2C). The term replication compartment is applicable to these structures because BrdU labeling was observed primarily within these domains (Fig. 2D). As indicated above, UL9 did not affect replication compartment formation (Fig. 2E), nor did it appear to inhibit the amplification of DNA at these sites (Fig. 2F). Therefore,
regardless of the presence of UL9, the other six essential replication proteins dramatically redistributed BrdU incorporation patterns, suggesting a functional association with DNA, likely cellular DNA.

To test if catalytic activity of the transiently expressed polymerase was necessary to promote expansion of prereplicative sites into replication compartments, parallel transfections were performed in the presence of PAA, a drug that specifically blocks the HSV polymerase but not cellular polymerases (Mao et al., 1975). PAA did inhibit viral compartment formation (Fig. 2G). Similar to the prereplicative sites observed within infected cells (de Bruyn Kops and Knipe, 1988), there was residual DNA synthesis occurring at the smaller clusters where ICP8 remained (Fig. 2H). This observed effect of PAA implies that the activity of HSV polymerase is uniquely required for enlargement of these punctate structures into replication compartments.

Prereplicative site heterogeneity and the effects of cellular DNA synthesis

The functional association of transfected viral proteins with DNA synthesis and our previous observation indicating a correlation between the state of cellular DNA synthesis and punctate structure formation in UL5\(^{-}\), UL8\(^{-}\), UL52\(^{-}\), and UL9\(^{-}\) mutant virus-infected cells (Liptak et al., 1996) prompted us to further characterize the effects of host-cell DNA replication on prereplicative site assembly in WT virus-infected cells. When asynchronous cell cultures were infected with WT virus in the presence of PAA, immunofluorescence staining for ICP8 at 5.5 hpi with the polyclonal 3-83 serum indicated that many cells contained 100 or more punctate structures, while another population of cells had only a few prereplicative sites (Fig. 3A). Labeling with BrdU for 30 min prior to fixation and double-staining for incorporated BrdU revealed a correlation between the replication state of the host cell and the number of prereplicative sites formed in each nucleus (Fig. 3, compare A and B). Within BrdU-positive cells, ICP8 accumulated at a large number of foci, many of which colocalized with the ongoing cellular DNA synthesis. Within cells not replicating DNA, however, only a limited number of punctate sites were present. These results suggested that cell DNA synthesis or some related S-phase factor stimulated the formation of at least some of these punctate structures and that distinct types of prereplicative sites may form within WT-infected cells in response to different conditions.

Inhibition of cellular S-phase restricts the number of viral prereplicative sites formed

To confirm that the state of the host cell exerts an effect on the number and possibly type of prereplicative sites formed, we compared viral replication structure assembly in cycling cells and cells chemically blocked in G1 by n-butyrate. Although the precise mechanism is not known, n-butyrate has been shown to cause G1 arrest...
FIG. 2. Colocalization of DNA synthesis with viral protein complexes in transfected cells. Various combinations of the HSV DNA replication genes were expressed in Vero cells by transient transfection. After overnight BrdU labeling, dual-staining immunofluorescence for ICP8 (3–83; left column) and incorporated BrdU (right column) was performed at 48 hr posttransfection. (A and B) ICP8; (C and D) ICP8 and helicase-primase plus polymerase (UL30 and UL42); (E and F) all seven replication genes; (G and H) all seven replication genes transfected in the presence of PAA.

in a wide variety of cell types including Vero cells (reviewed in Shadan et al., 1994). To verify that n-butyrate treatment prevented cellular DNA synthesis during these experiments, we incubated duplicate mock-infected cultures with BrdU 30 min prior to fixation. Immunofluorescence analysis of BrdU incorporation confirmed that while 59% of cells in untreated cultures were synthesizing DNA (Fig. 4A), treatment with n-butyrate efficiently suppressed BrdU incorporation to less than 1% (Fig. 4B). Parallel cultures of n-butyrate-treated and nontreated
cells were infected with WT virus or one of the replication-defective viruses, CgalΔ42 (UL42Δ) or hr80 (UL8Δ). At 5.5 hpi infected cells were processed for ICP8 immunofluorescence with 3-83 rabbit antiserum to determine the state of viral replication structure assembly. As expected, in the absence of any inhibitory drugs ICP8 was present in replication compartments in WT-infected Vero cells (Fig. 5A). Consistent with the report that n-butyrate does not inhibit HSV replication (Shadan et al., 1994), ICP8 was also detected in large replication compartments in n-butyrate-treated cells (Fig. 5B). Nevertheless, there was a striking difference between the prereplicative site patterns observed in n-butyrate-blocked cells and cycling cells. As shown in Fig. 3A, when WT infection was established in an asynchronous culture of cells and viral DNA synthesis was restricted, multiple prereplicative site patterns were observed. The same heterogeneity was noted in this experiment; however, because cell growth was fostered by infecting subconfluent cells and overlaying infections with DMEM–10%FCS rather than the usual 199 medium containing 1% FCS (see Materials and Methods), the majority of infected cells contained ICP8 at many punctate sites that filled the nucleus (Fig. 5C). None of the n-butyrate blocked cells infected in parallel exhibited this abundant prereplicative site pattern. Instead only a small number of punctate structures were detected in each nuclei, while much of the ICP8 remained diffusely distributed (Fig. 5D). The same n-butyrate effect on prereplicative site formation was seen if viral DNA replication was restricted by genetic mutation. Specifically, the abundant punctate sites observed in WT-infected cells in the presence of PAA were also detected in untreated cells

FIG. 3. Prereplicative site heterogeneity in WT virus-infected cells. Confluent Vero cells were infected with WT virus in the presence of PAA. BrdU labeling was performed from 5.5 to 6 hpi, and coverslips were processed for dual-label immunofluorescence. (A) ICP8 staining with 3–83 serum, (B) MAB BrdU staining, (C) phase-contrast image of the same field.

FIG. 4. Effect of n-butyrate on cellular DNA synthesis. Subconfluent Vero cells were incubated overnight in DMEM–10%FCS in the (A) absence or the (B) presence of 3 mM n-butyrate. BrdU labeling was performed 30 min before immunofluorescence staining with an anti-BrdU MAB.
FIG. 5. Prereplicative site formation in n-butyrate-arrested G1 cells. Vero cells were incubated with (right column) or without (left column) 3 mM n-butyrate overnight and subsequently infected under the same conditions with the indicated virus and inhibitor. ICP8 immunofluorescence was performed with 3-83 at 6 hpi. (A and B) WT; (C and D) WT + PAA; (E and F) CgalΔ42 (UL42⁺); (G and H) hr80 (UL8⁻) + PAA.

infected with the UL42 null mutant virus, CgalΔ42 (Fig. 5E). Likewise, the presence of n-butyrate restricted CgalΔ42 prereplicative site formation (Fig. 5F). As might be expected based on our previous observation of a correlation between active cellular DNA synthesis and punctate structure formation in helicase–primase mutant-infected cells (Liptak et al., 1996), the n-butyrate G1 arrest had an even more dramatic effect on prereplicative site assembly in cells infected with the UL8 null mutant hr80. Extensive intranuclear structure was detected in the cycling cells infected with hr80 in the presence of PAA (Fig. 5G); however, when n-butyrate-treated cells were infected with the same mutant under identical conditions, no punctate sites were observed (Fig. 5H).

Therefore, we could distinguish two species of prereplicative sites within infected cells. The formation of the
abundant variety was contingent upon active cellular DNA synthesis or some other S-phase host cell factor, while a smaller subset of prereplicative sites was independent of S-phase. Although the assembly of the latter type was not restricted to S-phase, it appeared to be more dependent on certain viral factors, as no S-phase-independent prereplicative sites were formed in the absence of UL8.

The two types of prereplicative sites formed within infected cells colocalize with distinct cellular proteins

To determine if the two kinds of punctate structures described above are assembled at different intranuclear locations and/or contain different cellular antigens, we monitored their localization relative to two types of nuclear structures. The correlation of the abundant class of prereplicative sites with S-phase suggested that it may be this particular species of punctate structures that occupies the same sites as the cellular replication machinery. In contrast, when infections were maintained in the presence of n-butyrate, the distributon of IC8 was observed to change. The localization pattern reported for cellular ND10 (Ascoli and Maul, 1991). To perform the correlation of the cellular replication apparatus (both active and inactive), we performed immunochemistry staining for the single-stranded DNA-binding protein RP-A. Visualization of cellular ND10 was accomplished with an antibody directed against the human autoantigen Sp100, which is one of the five proteins currently known to be present at these nuclear domains (Ascoli and Maul, 1991; Szostek et al., 1990).

In uninfected cells no IC8 was detected with the 3-83 serum (Fig. 6A) and most of the RP-A was distributed diffusely or in a fine granular pattern throughout the nucleus (Fig. 6B). As measured previously (Wilcock and Lane, 1991), dual-staining of WT-infected cells at 6 hpi revealed significant colocalization of cellular RP-A with viral replication structures. In the absence of inhibitory drugs, both IC8 and RP-A accumulated in globular replication compartments (Figs. 6C and 6D, respectively). RP-A (Fig. 6F) also became compartmentalized with IC8 (Fig. 6E) in many WT-infected cells in the presence of PAA. In particular, the subset of cells in which RP-A was associated with regions of IC8 accumulation included those that contained a large number of prereplicative sites. RP-A did not specifically localize with IC8 in the nuclei that formed only a limited number of structures (compare Figs. 6E and 6F). Infections done in the presence of n-butyrate showed restricted prereplicative site formation in all cells (Fig. 6G) and no redistribution of RP-A (Fig. 6H), confirming that it was the S-phase-independent species of assembly sites that failed to associate with cellular RP-A. Thus, only the S-phase-associated class of viral replication structures colocalized with RP-A.

Although the prereplicative sites in nonreplicating cells did not correspond with RP-A staining, their spatial pattern suggested that this may be a population of prereplicative sites that associate specifically with ND10. Because IC8 has been shown to disrupt ND10s (Maul et al., 1993; Everett and Maul, 1994; Maul and Everett, 1994), we infected n-butyrate-treated (Fig. 7) and noninfectected (data not shown) WI38 cells with WT virus or the IC8 null mutant virus, n212. Infections were incubated in the presence of PAA and processed for immunofluorescence at 6 hpi. The IC8 MAB 10-E3 showed no evidence of IC8 staining in uninfected cells (Fig. 7A), but cellular ND10s were detected with the Sp100 antibody (Fig. 7B).

As expected, n-butyrate-treated cells infected with WT virus showed IC8 accumulation at a small number of sites within individual nuclei (Fig. 7D); however, as expected, cellular ND10 structures were not present in these cells (Fig. 7E) because WT virus expresses IC80. In contrast, cells infected with the IC80-mutant virus, n212, allowed simultaneous detection of both IC8 and ND10. IC8 expressed by n212 in nonreplicating cells accumulated at limited numbers of prereplicative sites indistinguishable from those observed in WT-infected cells (Fig. 7G). Costaining of Sp100 showed a correspondence between IC8 localization and cellular ND10 structures (Fig. 7, compare G and H).

To further substantiate the apparent colocalization of these viral prereplicative sites with ND10, color micrographs were recorded from the rhodamine IC8 fluorescence (Figs. 8A and 8B) and fluorescein Sp100 staining (Figs. 8C and 8D) in n212-infected cells. All cells examined exhibited an association between this ND10 antigen and IC8 (Figs. 8C and 8D). Similar to the position of viral DNA relative to ND10 (Maul et al., 1996), the smaller ND10 structures detected in these cells overlapped areas of IC8, but were usually situated at the edge of the IC8-positive regions. Hence, in non-S-phase cells, one type of prereplicative site was formed and these almost exclusively were targeted to ND10, not sites of RP-A. In total, these indicates that the two varieties of prereplicative sites distinguished in these experiments preferentially assemble at different intranuclear substrutures and therefore contain unique cellular factors.

**DISCUSSION**

These studies were undertaken to investigate further the assembly of the HSV DNA replication proteins within the nucleus. The primary goal was to identify viral and cellular factors involved and determine why these requirements appear to vary under different conditions.

Formation of functional viral replication structures in transfected cells

We have continued to use transient transfection as a means to further define the viral proteins required for replication structure assembly. As shown previously (Liptak et al., 1996), neither viral origin-containing DNA nor the viral-encoded origin-binding protein is necessary for punctate structure formation outside the context of infec-
FIG. 6. Colocalization of S-phase-associated prereplicative sites with RP-A. Vero cells were infected under the conditions indicated below, and dual-label immunofluorescence with anti-ICP8 3-83 serum (left column) and anti-RP-A MAB p70-9 (right column) was performed at 6 hpi. (A and B) Mock; (C and D) WT; (E and F) WT + PAA; (G and H) n-butryrate-treated cells infected with WT + PAA.

Interestingly, the two distribution patterns of ICP8 observed in WT virus-infected cells which were indicative of different types of prereplicative sites were also present in cells transfected with ICP8 and the helicase-primase. Specifically, one-third of the transfected cells containing intranuclear structure were filled with prereplicative sites, while two-thirds contained a more limited number of complexes similar to what was observed in the non-replicating infected-cell nuclei. Therefore, based on the ICP8 patterns observed in transfected cells, viral proteins appear to be able to assemble at intranuclear sites associated with cellular DNA synthesis or ND10. This is consistent with recent data of Lukonis and Weller (1996) which demonstrate that in fact some viral structures formed in transfected cells do colocalize with ND10. Hence, it would appear that viral DNA is not required to
FIG. 7. Localization of S-phase-independent prereplicative sites relative to cellular ND10. Butyrate-treated WI38 cells were infected with WT or ICP0 null mutant virus, n212, at high m.o.i. (Vero cell equivalent of m.o.i. = 100). At 6 hpi dual-label immunofluorescence was performed to detect the localization of ICP8 (left column, A, D, and G) and the cellular ND10-associated protein Sp100 (middle column, B, E, and H). (A–C) Mock; (D–F) WT + PAA; (G–I) n212 + PAA.

tate structures formed. While previously no distinction was made among the viral-protein foci, collectively referred to as prereplicative sites, these complexes can be subdivided into two different classes which can be distinguished by several characteristics. One type of prereplicative site assembles in large numbers in S-phase cells and colocalizes with host DNA synthesis activity. Our previous observation that aphidicolin, like PAA, induces prereplicative site formation in cells infected with active viral polymerase (UL30) with its processivity factor (UL42) induces the dramatic movement of viral antigens from their initial cellular sites into globular replication structures. While no viral origin-containing DNA is needed, the association of viral proteins with DNA is evident from the fact that BrdU incorporation is detected within these large domains.

Assembly of viral proteins into two distinct types of prereplicative sites

Investigation of the effects of the host-cell state on viral replication structure assembly within virus-infected cells has allowed us to define more precisely the punc-
Colocalization of S-phase-independent prereplicative sites with cellular ND10. WI38 cells were pretreated with n-butyrate, infected with the ICP0 null mutant n212, and stained for ICP8 and Sp100 as described for Fig. 7. (A and D) Rhodamine fluorescence image of ICP8; (B and E) fluorescein fluorescence image of ND10 antigen Sp100; (C and F) double-exposure photographs showing the colocalization of ICP8 and Sp100 in (A and B) and (D and E), respectively.

Although viral DNA appears to be present only with the viral protein complexes assembled at ND10 (Ishov and Maul, 1996; Maul et al., 1996), all but one of the essential viral replication proteins (UL42) have been shown to be retained at the prevalent S-phase-associated structures (Quinlan et al., 1984; Olivo et al., 1989; Goodrich et al., 1990; Bush et al., 1991; Liptak et al., 1996; Lukonis and Weller, 1996). In addition, the BrdU redistribution and effect of PAA we observed in transfected cells suggests that viral proteins assembled at sites with DNA lacking viral origin sequences were capable of amplifying that DNA, just as the structures formed in association with ND10 appear to initiate viral replication. Therefore, all these foci are prereplicative sites where the viral antigens are deposited prior to replication, and all are ultimately capable of synthesizing DNA. However, the significance and function of each of these types of prereplicative sites within infected cells are most likely very different.

The significance of heterogeneity among prereplicative sites

The recognition that distinct species of prereplicative sites exist not only explains why we have detected different requirements for structure assembly under various conditions (Liptak et al., 1996), but also sheds new light on other past observations made about viral prereplicative sites. For example, it has been reported that host-cell DNA polymerase activity (de Bruyn Kops and Knipe, 1988) and related cellular factors (Wilcock and Lane, 1991) colocalize with viral prereplicative sites. Previously, the association of HSV proteins with the cellular replication apparatus has given the impression that viral replication might occur at modified cellular DNA synthesis sites, suggesting that some component(s) of the cellular machinery may be utilized by the virus. However, this colocalization with cellular DNA synthesis factors is in fact characteristic of the S-phase-associated prereplicative sites only. We now know that those abundant sites are distinct from the ND10-associated complexes that contain viral genomes (Maul et al., 1996). Thus, consistent with the fact that HSV replication is not contingent upon the host-cell cycle, viral DNA synthesis appears to initiate at S-phase-independent assemblies which do not contain a full complement of cellular replication factors. The localization of viral proteins to S-phase-associated structures may simply be due to nonproductive viral protein interactions with cellular DNA or some part of the cellular DNA synthesis apparatus. In this regard, the ability of HSV to arrest host cells in G1 (de Bruyn Kops and Knipe, 1988) may have evolved to prevent cells from entering S-phase which might limit the amount of viral replication proteins that become "misdirected" to sites of cellular DNA synthesis at ND10.
DNA synthesis. Alternatively, the presence of viral proteins at cellular DNA synthesis sites may serve a specific purpose, such as inducing the release of cellular replication factors that are needed elsewhere by the virus.

While it has been shown previously that a small fraction of ICP8 in infected cells tends to colocalize with ND10 (Maul et al., 1996), the identification of a particular subset of prereplicative sites associated with ND10 provides the missing link between the distribution patterns historically observed for viral replication proteins and the more recently reported localization of viral DNA to ND10 (Ishov and Maul, 1996; Maul et al., 1996). Because viral genomes are preferentially targeted to sites juxtaposed to ND10 and replication compartments appear to expand from those points, it seems likely that viral replication begins at the particular subset of prereplicative sites that form there. Importantly, this distinction among prereplicative structures could prove to be useful in defining any cellular factors required for HSV DNA synthesis. Being able to focus on these "pre"-replication compartment complexes (i.e., structures formed in the presence of n-butylate), should make it easier to identify cellular proteins that are specifically recruited to viral replication sites during viral infection.

Because all known ND10-associated proteins are dispersed immediately upon infection with viruses expressing the immediate-early gene product, ICP0 (Maul et al., 1993; Everett and Maul, 1994; Maul and Everett, 1994), it is not clear that ND10s are the actual structures with which viral proteins interact. There may be some other, yet unidentified, cellular structure situated closely to ND10. Another possibility is that an unknown component(s) of ND10 is not disrupted by viral infection, leaving some form of intact ND10 available as a binding site for viral factors. In any event, further investigation of viral DNA and protein localization to these intranuclear sites could shed light on viral replication structure assembly, viral-host interactions, and any protein signals needed for targeting to these sites.

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Note added in proof. The two types of prereplicative site patterns defined in this work through the use of n-butylate are also observed in synchronized cell populations in the absence of drugs. Specifically, abundant prereplicative sites are observed in synchronized S-phase cells while the less abundant sites are observed in G1-phase cells (A. de Bruyn Kops, 1991, Ph.D. thesis, Harvard University, Cambridge, MA).

REFERENCES


