Formation of DNA Replication Structures in Herpes Virus-Infected Cells Requires a Viral DNA Binding Protein

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Summary

Eukaryotic DNA synthesis is thought to occur in multi-enzyme complexes present at numerous discrete sites throughout the nucleus. We demonstrate here that cellular DNA replication sites identified by bromodeoxyuridine labeling are relocated in cells infected with herpes simplex virus such that they correspond to viral prereplicative structures containing the HSV DNA replication protein, ICP8. Thus components of the cellular DNA replication apparatus are present at viral prereplicative sites. Mutant virus strains expressing defective ICP8 do not alter the pattern of host cell DNA replication sites, indicating that functional ICP8 is required for the redistribution of cellular DNA replication complexes. This demonstrates that a specific protein molecule can play a role in the organization of DNA replication proteins at discrete sites within the cell nucleus.

Introduction

Complex nuclear processes such as DNA replication, transcription and RNA processing may rely on a high level of intranuclear organization for their efficient execution in eukaryotic cells. Functional complexes of proteins and nucleic acids along with nonchromatin proteinaceous structures (reviewed by Agutter and Richardson, 1980) can be envisioned as contributing to the ordering of such processes within eukaryotic cell nuclei. The dependence of mRNA splicing on the formation of ribonucleoprotein complexes referred to as spliceosomes (reviewed by Padgett et al., 1986) provides a precedent for the idea that certain nuclear processes are carried out by multi-component functional complexes. Recent biochemical and genetic evidence that many of the enzymes involved in eukaryotic DNA replication are present in complexes known as replication complexes (reviewed by Huberman, 1987) suggests that DNA synthesis may also be facilitated by the organization of proteins into multi-subunit assemblies. However, the spatial arrangement of the eukaryotic DNA replication apparatus within the nucleus and the way in which DNA replication proteins are assembled into functional complexes is not yet understood.

Support for the idea that cellular DNA replication proteins are organized at particular locations in the nucleus has been obtained using thymidine autoradiography (Huberman et al., 1973; Pardoll et al., 1980) and bromodeoxyuridine (BrdU) immunofluorescence microscopy (Nakamura et al., 1986) to locate newly synthesized DNA in mammalian cells. These and other studies (Dijkwel et al., 1979; Vogelstein et al., 1980; McCreary et al., 1980; Berzney and Bucholtz, 1981a; Cook and Brazzolli, 1980) have shown that DNA replication occurs at numerous discrete sites throughout the nucleus where DNA is attached to a detergent-insoluble nuclear framework or matrix (Berezney and Coffey, 1975, 1977; Berezney and Bucholtz, 1981b; Capco et al., 1982). However, the nature of these matrix-associated DNA replication sites remains obscure because the composition of extracted matrices and their correlation with nuclear substructure in vivo has been difficult to establish.

Viral systems have been useful in probing a variety of eukaryotic processes, including protein synthesis, targeting, and subunit assembly. Cells infected with herpes simplex virus organize viral proteins at intranuclear sites related to viral DNA synthesis (Quinlan et al., 1984) and thus provide a system for studying the assembly of DNA replication protein complexes. Transfection experiments and analyses of temperature-sensitive HSV-1 mutants have identified seven herpes gene products needed for viral DNA replication, including the HSV DNA polymerase and the multi-functional DNA binding protein, Infected Cell Protein 8 (ICP8) (Powell and Purifoy, 1977, Jofre et al., 1977; Chu et al., 1979; Conley et al., 1981; Matz et al., 1983; Chalberg, 1986; Wu et al., 1988; McGeoch et al., 1988; Marchetti et al., 1988). ICP8 and some of the other HSV replication proteins are possible components of viral DNA replication complexes.

ICP8 is localized to the host nucleus in a maturation process involving the association of the protein with the nuclear framework and, ultimately, its binding to viral DNA (Fenwick et al., 1978; Knipe and Spang, 1982; Quinlan and Knipe, 1983; Lee and Knipe, 1983). In addition, the intranuclear distribution of ICP8 in infected cells is dependent on the status of viral DNA replication (Quinlan et al., 1984). When viral DNA replication occurs, ICP8 accumulates in large, globular replication compartments where it is associated with viral DNA (Quinlan et al., 1984). When HSV DNA replication is blocked by treatment with sodium phosphonoacetate, which inhibits the viral DNA polymerase (Mao and Robishaw, 1975), or when replication is absent during infection with a polymerase-defective mutant, ICP8 is detected by immunofluorescence at numerous loci called prereplicative sites (Quinlan et al., 1984). Under these conditions, ICP8 is associated with a nuclear protein framework (Quinlan et al., 1984). ICP8 redistributes from prereplicative sites to replication compartments when viral DNA replication is permitted, but returns to the prereplicative sites when viral DNA synthesis is inhibited (Quinlan et al., 1984). It is not yet known whether the prereplicative sites are intermediates in the formation of the replication compartments.

The ability of ICP8 to bind to both DNA and the nuclear substructure, along with its requirement for viral DNA replication, raises the possibility that this large, multi-functional protein might play a role in organizing cellular...
and viral components into structures related to HSV DNA replication. Here we demonstrate that the distribution of cellular DNA replication sites is altered in HSV-infected cells such that it corresponds with the pattern of viral prereplicative sites. Using HSV mutants, we show that functional ICP8 is required for the formation of the prereplicative sites. The assay used should be applicable to other HSV mutants with altered DNA replication proteins to define a viral protein assembly map for these nuclear structures. Viral proteins able to affect the organization of the cellular DNA replication apparatus could provide paradigms for cellular proteins involved in the assembly of nuclear structures.

Results

To investigate the possible role of the HSV major DNA binding protein, ICP8, in the assembly of DNA replication structures, we have compared the intranuclear distributions of ICPR and DNA replication sites in cells infected with wild-type or mutant viruses. The recent development of antibodies recognizing the thymidine analog, bromodeoxyuridine (BrdU) (Gratzner, 1982), has permitted the use of BrdU labeling coupled with immunofluorescence microscopy to reveal the distribution of DNA replication sites within cell nuclei (Nakamura et al., 1986). The technique offers improved resolution over autoradiographic methods and facilitates simultaneous localization of other nuclear antigens. BrdU labeling and indirect immunofluorescence techniques were used in this study to identify sites of DNA replication in uninfected and HSV-infected monkey CV-1 cell monolayer cultures.

Cellular DNA Replication Occurs at Viral Prereplicative Sites

To determine the effects of HSV-1 infection on the distribution of cellular DNA replication sites, CV-1 cells were mock-infected or infected with wild-type virus in the presence of sodium phosphonoacetate (PAA), a compound that preferentially inhibits the HSV-1 DNA polymerase. Cells were labeled with BrdU for 15 min immediately prior to fixation at 5 hr post infection (hpi) and stained with both anti-ICP8 serum and anti-BrdU antibody followed by FITC-conjugated goat anti-mouse and RITC-conjugated goat anti-rabbit immunoglobulins.

BrdU staining in the majority of mock-infected cells (Figure 1A) or uninfected cells (not shown) treated with PAA revealed a fine granular pattern of DNA replication sites. This pattern of BrdU staining was similar to that observed in uninfected cells by others (Nakamura et al., 1986) and consistent with autoradiographic data suggesting that cellular DNA replication occurs at numerous discrete sites in...
HSV ICP8 and DNA Replication Structures

Figure 2. Cellular DNA Replication in Cells Infected during G1 Phase or S Phase of the Cell Cycle

Cells were synchronized by mitotic shake-off and replated in medium containing 10% serum. Uninfected cells were labeled with BrdU and processed for immunofluorescence at 7 hr (A) or 13.5 hr (B) after replating. Cells mock-infected (C, D) or infected with KOS1.1 (E, F) at 7 hr (C, E) or 13.5 hr (D, F) in the presence of PAA were labeled with BrdU and processed for immunofluorescence at 5 hpi. Cells were then stained for BrdU as described in the legend to Figure 1. (A–F) BrdU immunofluorescence micrographs. Bar in (A) equals 20 μm in (A) and (B). Bar in (C) equals 10 μm in (C–F).

the nucleus (Huberman et al., 1973; Pardoll et al., 1980). In contrast, cellular DNA replication sites identified by BrdU labeling in cells infected in the presence of PAA (Figure 1C) were larger and fewer in number than the fine granular sites in mock-infected cells (Figure 1A). In addition, the cellular DNA replication sites in infected cells co-distributed with ICP8 (Figure 1D) in the previously described viral prereplicative sites (Quinlan et al., 1984).

To permit recognition of BrdU-substituted DNA by the anti-BrdU antibody, the cells used in these experiments were treated with HCl following fixation in formaldehyde and permeabilization in acetone. Acid treatment did not affect the distribution of ICP8 in prereplicative sites. Furthermore, the co-distribution of ICP8 and BrdU at prereplicative sites appears to reflect the specific organization of DNA replication proteins rather than a general aggregation of nuclear antigens because HSV-1 nuclear proteins such as ICP4 and ICP27, which are not directly required for viral DNA replication (Challberg, 1986), did not accumulate at prereplicative sites (A. de Bruyn Kops and D. Knipe, unpublished data).

Distribution of Cellular DNA Replication Sites in G1 or S Phase Cells

The presence of cellular DNA replication at viral prereplicative sites indicated that components of the cell DNA replication apparatus are organized at these locations. This suggested that cellular DNA replication complexes formed prior to infection may be incorporated into viral prereplicative structures. Alternatively, cell and viral DNA replication proteins may be assembled de novo into viral structures capable of synthesizing cellular DNA. To distinguish between these two possibilities, the pattern of cellular DNA replication sites was compared in synchronized CV-1 cells infected during G1 or S phases of the cell cycle using BrdU immunofluorescence.
Figure 3. Time Course of Prereplicative Structure Formation

CV-1 cells were infected with HSV-1 strain KOS1.1 at 37°C in the presence of PAA. Cells were labeled with BrdU, fixed at 0 (A, B), 3 (C, D), 4 (E, F), or 6 (G, H) hpi and reacted with anti-BrdU and anti-ICP8 antibodies as in Figure 1. (A, C, E, G) ICP8 immunofluorescence micrographs. (B, D, E, F) BrdU immunofluorescence micrographs. Bar equals 10 μm.
[3H]thymidine labeling of CV-1 cells synchronized using standard mitotic shake-off techniques showed that cells entered S phase 8–10 hr after replating; S phase lasted for about 9 hr with maximum incorporation of label occurring between 14–18 hr after replating (data not shown). Consistent with this, uninfected cells labeled with BrdU for 15 min at 7 hr after replating (G1 phase) showed incorporation of label in only 5% of the cells (Figure 2A), while approximately 75% of the cells incorporated BrdU at 13.5 hr (S phase) after replating (Figure 2B).

To examine the distribution of cellular DNA replication sites in cells infected during the G1 or S phase, synchronized cell cultures were mock infected or infected in the presence of PAA at 7 hr (G1 phase) or 13.5 hr (S phase) after replating. Approximately 50% of cells mock infected in G1 phase (Figure 2C) and 75% of cells mock infected in S phase (Figure 2D) incorporated BrdU when labeled at 5 hpi. Similarly, 75% of cells infected in S phase incorporated BrdU at 5 hpi. The distribution of cellular DNA replication sites under the last conditions (Figure 2F) was altered from the pattern in mock-infected cells (Figure 2D) and resembled the prereplicative site pattern observed in asynchronous cell cultures (Figure 1). In contrast, cells infected during G1 phase and labeled at 5 hpi showed incorporation of BrdU in less than 12% of the cells (Figure 2E). As with unsynchronized cells, ICP8 was distributed in prereplicative sites in cells infected in the presence of PAA in G1 or G0 phases of the cell cycle (data not shown).

The simplest interpretation of these results is that while cells infected during S phase synthesize cellular DNA at prereplicative sites, the majority of cells infected during G1 phase do not progress into S phase. Therefore, unsynchronized cells that incorporated BrdU at viral prereplicative sites (Figure 1) must have formed cellular DNA replication complexes and entered S phase prior to or very early during infection. This suggests that preexisting cellular DNA replication complexes were redistributed to the viral prereplicative sites in these cells. These results do not rule out the possibility that components of the cell DNA replication apparatus insufficient to support DNA synthesis were incorporated into prereplicative sites in cells infected during the G1 phase of the cell cycle in the presence of PAA.

Kinetics of Prereplicative Site Formation

To compare the kinetics of prereplicative site formation with the redistribution of cellular DNA replication sites, cells were infected in the presence of PAA and labeled with BrdU for 15 min prior to fixation at 3, 4, or 6 hpi. The cells were processed for immunofluorescence and stained with both anti-ICP8 serum and anti-BrdU antibody as described above. At early times after infection (3 hpi), ICP8 staining was evident at a few discrete nuclear foci (Figure 3C). ICP8 appeared at an increased number of foci by 4 hpi (Figure 3E) and was evident in the previously characterized pattern of prereplicative sites by 6 hpi (Figure 3G). At 3 hpi, when ICP8 was concentrated at a few sites, BrdU staining in most of the infected cells (Figure 3D) was indistinguishable from that observed in mock-infected cells (Figure 3B). As with mock-infected cells, a fraction of the cells infected in the presence of PAA showed no BrdU incorporation (Figure 3D) and likely lacked cellular as well as viral DNA synthesis. As more ICP8 accumulated in viral prereplicative sites (4 hpi), BrdU labeling became progressively restricted to these same sites (Figure 3F).

By 5–6 hpi, the sites of BrdU incorporation corresponded with the prereplicative sites (Figures 3G and 3H) as described above. Thus, the formation of viral prereplicative sites involved both the accumulation of ICP8 and the redistribution of cellular DNA replication centers to specific intranuclear sites.

Stages in the Formation of Replication Compartments

To determine whether formation of replication compartments in cells infected in the absence of PAA (Quinlan et al., 1984) involved stages similar to those seen during prereplicative site formation (Figure 3), ICP8 and BrdU distributions were examined in cells infected for 3, 4, or 6 hr without PAA. At early times after infection (3 hpi), ICP8 was apparent at a few foci (Figure 4C). This distribution was quite similar to that observed at 3 hpi in the presence of PAA (Figure 3C). Indicating that ICP8 localization up to this time was independent of the ability of the viral polymerase to replicate DNA. Between approximately 3 and 6 hpi, the ICP8 patterns observed in PAA-treated and untreated cells became increasingly different. In untreated cells, ICP8 continued to accumulate at a few locations in the nucleus (Figure 4E), leading to the pattern of large replication compartments by 6 hpi (Figure 4F) instead of the prereplicative site distribution described above.

The granular patterns of cellular DNA replication sites revealed by BrdU staining in PAA-treated (Figure 3B) or untreated (Figure 4B) cells after mock-infection were indistinguishable. In addition, the majority of cells infected for 3 hr in the absence of PAA also showed a similar granular distribution of BrdU incorporation (Figure 4D). In contrast, the BrdU staining pattern in cells infected for longer times (4 hr) in the absence of PAA (Figure 4F) corresponded increasingly with the distribution of ICP8 (Figure 4E). By 6 hpi, all BrdU staining (Figure 4H) co-distributed with ICP8 in replication compartments (Figure 4G). This was consistent with earlier studies in which [3H] thymidine (Rixon et al., 1983) and BrdU (Martin et al., 1987) labeling identified areas of DNA replication resembling replication compartments in the nuclei of HSV-infected cells.

In contrast to the absence of BrdU incorporation in some infected cells treated with PAA, all untreated infected cells incorporated BrdU. However, in some cells at early times post infection (3 hpi), the BrdU label was restricted to foci of ICP8 accumulation (Figures 4C and 4D). Given that this limited BrdU staining pattern was never observed in PAA-treated cells, the incorporation of label at the ICP8 foci in untreated cells most likely represented viral DNA synthesis. The incorporation of BrdU at the early ICP8 foci suggests that these sites are the earliest locations of viral DNA replication in the infected cells.

BrdU Staining Represents De Novo DNA Synthesis

The assumption that BrdU labeling in these experiments represented de novo DNA replication was supported by the ability of the drug aphidicolin to eliminate BrdU stain-
Figure 4. Time Course of Replication Compartment Formation

CV-1 cells were infected with HSV-1 strain KOS1.1 at 37°C in the absence of PAA. Cells were labeled with BrdU and fixed at 0 (A, B), 3 (C, D), 4 (E, F), and 6 (G, H) hpi. Cells were processed for immunofluorescence and reacted with anti-BrdU and 3-x anti-ICP8 antibodies as in Figure 1. (A, C, E, G) ICP8 immunofluorescence micrographs. (B, D, F, H) BrdU immunofluorescence micrographs. Bar equals 10 μm.
Table 1 [3H]Thymidine Incorporation into Viral and Cellular DNA in HSV-1-infected and Mock-infected Cells

<table>
<thead>
<tr>
<th>Infection</th>
<th>Cellular DNA(^b) (CPM)</th>
<th>HSV DNA(^a) (CPM)</th>
</tr>
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<tbody>
<tr>
<td>mock</td>
<td>86,300</td>
<td>&lt;100</td>
</tr>
<tr>
<td>PAA(^c)</td>
<td>32,500</td>
<td>&lt;100</td>
</tr>
<tr>
<td>+ APH(^d)</td>
<td>900</td>
<td>&lt;100</td>
</tr>
<tr>
<td>KOS1.1</td>
<td>58,100</td>
<td>56,200</td>
</tr>
<tr>
<td>+ PAA</td>
<td>14,100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>+ APH</td>
<td>2,600</td>
<td>&lt;100</td>
</tr>
</tbody>
</table>

\(^a\) DNA of densities 1.535–1.555 g/cm\(^3\).
\(^b\) DNA of densities 1.560–1.580 g/cm\(^3\).
\(^c\) dithiopyrimidine (400 µg/ml).
\(^d\) aphidicolin (20 µg/ml).

Thus, the distribution of cellular DNA replication sites that changed during infection with two different temperature-sensitive mutants encoding an altered ICP8 protein, we determined whether functional ICP8 was required for the formation of the prereplicative sites. To evaluate whether HSV-1 strains expressing defective forms of ICP8 could form prereplicative sites and redistribute cellular DNA replication, we examined the pattern of BrdU staining in cells infected with the temperature-sensitive mutants KOS tsD9, KOS1.1 ts13 or KOS1.1 ts18. The complementation group 1–1 mutants ts13 and ts18 express altered forms of ICP8 which do not accumulate in replication compartments at the nonpermissive temperature (Villarreal and Knipe, unpublished data). Other studies have shown that complementation group 1–1 mutants (Schafer et al., 1973) are defective for viral DNA replication at the nonpermissive temperature (Conley et al., 1981; Holland et al., 1984; Leinbach et al., 1984; Weller et al., 1983). The tsD9 mutant expresses normal ICP8 but encodes a DNA polymerase that is defective at the nonpermissive temperature (Jofre et al., 1977).

Cells infected with ts13 were stained with both the anti-ICP8 polyclonal rabbit serum and the anti-BrdU mouse monoclonal antibody as described previously. The anti-ICP8 polyclonal antiserum does not recognize the mutant ICP8 expressed by ts18. Therefore, cells infected with ts18 were stained separately with the 10-E3 anti-ICP8 mouse monoclonal antibody and the anti-BrdU monoclonal antibody followed by rhodamine-conjugated goat anti-mouse serum. In cells infected with ts13 at 39.7°C in the presence of PAA, the mutant ICP8 entered the nucleus but did not accumulate in typical prereplicative sites (Figure 5E). Instead, its distribution was diffuse, with some irregular concentrations of protein that were different from the wild-type prereplicative sites. Under the same conditions, the distribution of ICP6 produced by ts18 was primarily perinuclear (Figure 5G). The altered ICP8 distributions were specific for the mutants and not a general effect of incubation at 39.7°C because ICP8 expressed by wild-type virus was observed in prereplicative sites under these conditions (Figure 5A). Similarly, ICP8 expressed in cells infected with the HSV-1 DNA polymerase mutant KOS tsD9 at 39.7°C in the presence of PAA accumulated in prereplicative sites (Figure 5C) as reported previously (Quinan et al., 1984).

The sites of BrdU staining in cells infected with wild type or tsD9 virus at 39.7°C corresponded to the prereplicative sites containing ICP6 (Figures 5A–5D). Strikingly, the pattern of BrdU staining in ts13- or ts18-infected cells under these conditions was indistinguishable from that observed in mock-infected cells (Figures 5F, 5H, and 5J). Thus, the distribution of cellular DNA replication sites that was altered by wild-type virus infection remained unchanged during infection with two different temperature-sensitive ICP8 mutants. In addition, cells infected with an ICP8 gene deletion mutant, KOS1.1 Δ301 (M. Gao and D. Knipe, unpublished data), also failed to rearrange sites of cellular DNA replication (A. de Bruyn Kops and D. Knipe, unpublished data).
Figure 5. Comparison of ICP8 and BrdU Distribution in Mutant and Wild-type HSV-Infected Cells

CV-1 cells were infected with HSV-1 strains KOS1.1 (A and B), KOS tsD9 (C, D), KOS1.1 ts13 (E, F), or KOS1.1 ts18 (G, H) or mock-infected (I, J). Infections were carried out at 39.7°C in the presence of PAA. Cells were labeled with BrdU and fixed at 6 hpi. Cells were processed for immunofluorescence as in Figure 1. Cells shown in (A-F), (I), and (J) were reacted with anti-BrdU and 3R3 anti-ICP8 antibodies followed by RITC-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit immunoglobulins. Cells shown in (G) and (H) were reacted with anti-BrdU and 10E3 anti-ICP8 (Rose et al., 1986) antibodies, respectively, followed by RITC-conjugated goat anti-mouse immunoglobulins. (A, C, E, G, I) ICP8 immunofluorescence micrographs. (B, D, F, H, J) BrdU immunofluorescence micrographs. Bar equals 10 µm.
unpublished data). These results indicate that functional ICP8 was required for the virus-induced formation of prereplicative sites and the corresponding rearrangement of cellular DNA replication sites. In contrast, functional HSV DNA polymerase was not required for the localization of ICP8 (Quinlan et al., 1984) or the redistribution of host DNA replication centers.

**Discussion**

We have previously shown that the herpes simplex virus DNA replication protein, ICP8, accumulates at specific locations within the cell nucleus by 4–5 hr post infection (hpi) (Quinlan et al., 1984). Under conditions where HSV DNA synthesis is permitted, ICP8 is bound to viral DNA and localized in large, globular replication compartments. When viral DNA replication is inhibited, ICP8 accumulates at more numerous locations known as prereplicative sites, where it is associated with a nuclear protein framework (Quinlan et al., 1984). In this work we present results which indicate that the HSV prereplicative sites are virus-induced structures incorporating both cellular and HSV DNA replication proteins and that ICP8 plays a key role in their formation.

**Formation of HSV DNA Replicative Structures**

Our kinetic studies further define the ICP8 localization pathway by showing that ICP8 accumulates initially at a few discrete foci in the nuclei of infected cells. Depending on whether or not viral DNA synthesis is permitted, these early ICP8 foci appear to evolve into replication compartments or increase in number to give the prereplicative site pattern. BrdU incorporation at the early ICP8 foci in infected cells where cellular DNA synthesis appears to be absent suggests that the foci may define the earliest sites of HSV DNA synthesis. We hypothesize that viral DNA synthesis initiates within the early foci and that they grow into the large replication compartments as HSV DNA replication proceeds. The development of early foci into replication compartments may require viral DNA synthesis and the accumulation of progeny DNA at these sites. The prereplicative site pattern observed when viral DNA replication is inhibited may reflect the continued accumulation of DNA replication proteins at additional early foci. Although we do not yet know whether the individual prereplicative sites are equivalent to the foci observed at early times, the available data are consistent with this hypothesis.

**Prereplicative Sites Represent Virus-Induced Structures**

The observation reported here, that in infected cells treated with PAA, cellular DNA replication is redistributed to prereplicative sites not seen in mock-infected cells, indicates that the prereplicative sites represent novel structures induced by HSV infection. We do not yet know the extent to which the location of the prereplicative sites is determined by preexisting nuclear structures; however, the dramatically altered pattern of cell DNA replication sites in infected cells indicates that components of the cellular DNA replication apparatus are redistributed as a result of HSV infection. The presence of the HSV DNA polymerase at these same locations (M. Bush, D. Yager, M. Gao, A. Irmieri, D. Coen, and D. Knipe, unpublished data) suggests that the prereplicative sites previously defined by ICP8 immunofluorescence represent novel structures containing both viral and cellular DNA replication proteins. Based on the observation that cells infected during the G1 phase of the cell cycle do not progress into S phase, we favor the model that cellular DNA replication complexes formed prior to infection are incorporated into the viral prereplicative structures. The presence of cellular DNA replication complexes at virus-induced DNA replication structures may provide cellular factors needed for efficient HSV DNA synthesis.

The organization of viral DNA replication proteins such as ICP8 and polymerase at discrete sites in the cell nucleus suggests that there are nuclear domains organized for HSV DNA synthesis, just as there are locations in the normal cell nucleus where cellular DNA synthesis occurs (Huberman, 1987). The assembly of HSV proteins into prereplicative structures provides a system for the study of nuclear organization of DNA replication proteins and may make it possible to test whether the localization of certain proteins to specific nuclear sites is necessary for efficient DNA replication.

**ICP8 Has an Organizational Function**

In contrast to cells infected with wild-type HSV, cells infected with HSV strains encoding a defective ICP8 protein do not show redistribution of cellular DNA synthesis sites, which indicate that functional ICP8 is needed for this change in the infected cell. Because ICP8 is both present at the prereplicative sites and required for the redistribution of cell DNA replication sites, we hypothesize that ICP8 plays a direct role in the reorganization of the cell nucleus or maintenance of the reorganized state. ICP8 and other HSV proteins may localize to preexisting cellular DNA replication complexes and cause their conversion to viral prereplicative structures. Alternatively, ICP8 may accumulate at locations distinct from sites of cell DNA replication and act in concert with other viral proteins to recruit cellular DNA replication protein complexes to the new locations.

In addition to their inability to redistribute components of the cellular DNA replication apparatus, the ICP8-defective HSV strains also fail to accumulate the viral DNA polymerase at prereplicative sites (M. Bush, D. Yager, M. Gao, A. Irmieri, D. Coen and D. Knipe, unpublished data). This indicates that these mutants do not form the typical HSV structures at prereplicative sites. ICP8 mutants have previously been shown to be defective in DNA replication (Conley et al., 1981; Holland et al., 1984; Leinbach et al., 1984). Two important hypotheses are suggested by these observations: One, accumulation of the viral proteins and certain cellular components at the prereplicative sites may reflect the assembly of complexes in preparation for viral DNA replication. Two, localization of HSV DNA replication proteins to specific nuclear sites may be an important, if not essential, step in viral DNA synthesis.
Most models concerning the function of ICP8 in viral DNA replication have been based on in vitro studies and have focused on the protein's DNA binding properties. Huyechan (1989) observed a limited stimulation of the HSV DNA polymerase by ICP8 in a reaction containing a single-stranded DNA template. In contrast, O'Donnell et al. (1987) found that ICP8 inhibited the HSV DNA polymerase on a single-stranded DNA template but stimulated the HSV DNA polymerase on a double-stranded DNA template in the presence of other infected-cell proteins. The requirement for infected-cell extract in this system suggests that other viral and possibly cellular proteins may be needed for ICP8 to exert a stimulatory effect on viral DNA synthesis. This is consistent with our model that ICP8 has a role in organizing viral and cellular DNA replication proteins in the cell nucleus in addition to binding to DNA. Chiou et al. (1985) have provided genetic data indicating a possible interaction between ICP8 and the HSV DNA polymerase. Their results show that mutations in the ICP8 gene can affect the sensitivity of the virus to antiviral compounds that inhibit the HSV DNA polymerase. This alteration in sensitivity could be mediated by a direct interaction between ICP8 and the polymerase.

The genetic approach used in this study should allow us to determine whether other HSV proteins (Challberg, 1986; Wu et al., 1988) are required for assembly of viral replicative structures and whether a specific spatial arrangement of DNA replication proteins in the infected cell nucleus is necessary for efficient HSV DNA replication in vivo. The work described here showing that ICP8 plays a role in organizing DNA replication proteins within the cell nucleus demonstrates that a specific protein can play an organizational role with regard to DNA replication complexes. ICP8 may provide a paradigm of proteins that interact with the structural framework of the cell nucleus and play roles in defining the intranuclear localization of DNA replication proteins.

Experimental Procedures

Cells and Viruses

Monkey CV-1 (ATCC) cells were used for infections. Cells were grown on 12 mm circular glass cover slips in DME medium (Irvin Scientific) containing 10% inactivated fetal calf serum. HSV type 1 strains KOS1.l, KOS rDI (Schaffer et al., 1973), KOS1.l.1 ts13, and KOS1.l ts18 (Holland et al., 1984) were grown and titered as previously described (Knipe et al., 1987) antibody were used to detect ICP8.

Materials

Bromodeoxyuridine (Sigma), [3H]thymidine (New England Nuclear), aphidicolin (Calbiochem), disodium phosphonoacetate (a gift from Abbott Laboratories), pronase (Calbiochem), glycerol-gelatin (Sigma), and p-phenyldiamine (Sigma) were obtained from the indicated suppliers. Anti-ICP8 mouse monoclonal antibody was purchased from Cappel Laboratories. RITC-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit antibodies were spread on each cover slip. Singly stained cover slips were covered with 10 μl of a 1:100 dilution of RITC-conjugated goat anti-mouse antibody. The cover slips were then washed three times for 5 min in PBS. Ten microliters of PBS containing a 1:100 dilution of FITC-conjugated goat anti-rabbit antibodies was then added to each double-stained cover slip. Singly stained cover slips were covered with 10 μl of a 1:100 dilution of FITC-conjugated goat anti-rabbit antibody. The cover slips were then washed three times for 5 min in PBS. Ten microliters of PBS containing a 1:100 dilution of RITC-conjugated goat anti-mouse antibody. The cover slips were then washed three times for 5 min in PBS.

Indirect Immunofluorescence

Cells were grown on cover slips and infected with virus in the presence or absence of 400 μg/ml phosphonoacetate. Cells were labeled with BrdU for 15 min prior to fixation with 2% formaldehyde in phosphate-buffered saline (PBS), pH 7.6. The cover slips were rinsed with PBS and then with glass-distilled water, and the cells were permeabilized with acetone for 5 min at -20°C. The cells were rinsed with glass-distilled water and treated for 10 min with 4 N HCl to expose the incorporated BrdU residues. The cells were washed two times for 5 min in PBS to remove the acid. For dual staining, 10 μl of PBS containing a 1:10 dilution of the anti-ICP8 serum and a 1:60 dilution of the 3 83 anti-ICP8 antibody were spread on each cover slip. For single staining, 10 μl of a 1:30 dilution of the 10 E3 anti-ICP8 monoclonal antibody in PBS was added to each cover slip. The cover slips were incubated for 30 min at 37°C in a humid chamber. They were then washed three times for 5 min in PBS. Ten microliters of PBS containing a 1:100 dilution of RITC-conjugated goat anti-mouse antibody. The cover slips were then washed three times for 5 min in PBS. Ten microliters of PBS containing a 1:100 dilution of RITC-conjugated goat anti-mouse antibody. The cover slips were then washed three times for 5 min in PBS. Ten microliters of PBS containing a 1:100 dilution of FITC-conjugated goat anti-rabbit antibodies was then added to each double-stained cover slip. Singly stained cover slips were covered with 10 μl of a 1:100 dilution of FITC-conjugated goat anti-rabbit antibody. The cover slips were then washed three times for 5 min in PBS. Ten microliters of PBS containing a 1:100 dilution of RITC-conjugated goat anti-mouse antibody. The cover slips were then washed three times for 5 min in PBS. Ten microliters of PBS containing a 1:100 dilution of FITC-conjugated goat anti-rabbit antibodies was then added to each double-stained cover slip. Singly stained cover slips were covered with 10 μl of a 1:100 dilution of FITC-conjugated goat anti-rabbit antibody. The cover slips were then washed three times for 5 min in PBS.


