Comparison of the Intranuclear Distributions of Herpes Simplex Virus Proteins Involved in Various Viral Functions

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Received July 23, 1998; returned to author for revision September 3, 1998; accepted September 29, 1998

Herpesviral transcription, DNA synthesis, and capsid assembly occur within the infected cell nucleus. To further define the spatial relationship among these processes, we have examined the intranuclear distributions of viral DNA replication, gene regulatory, and capsid proteins using dual label immunofluorescence and confocal microscopy. We observed that several of the viral DNA replication proteins localize preferentially to punctate structures within replication compartments while the major transcriptional activator, ICP4, and the ICP27 regulatory protein show a more diffuse distribution within replication compartments. The viral proteins that show a punctate distribution in replication compartments redistribute from these compartments to prereplicative sites when viral DNA replication is inhibited, whereas viral proteins that show a diffuse distribution remain within replication compartments when viral DNA replication is inhibited. Thus the sites of viral DNA replication and late transcription appear to be distinct but codistribute within the boundaries of replication compartments. The major capsid protein, ICP5, also localizes initially to a diffuse distribution within replication compartments, but during the time of maximal progeny virus assembly, ICP5 becomes localized to punctate structures within replication compartments that are often near the punctate structures occupied by viral DNA replication proteins. Hence the processes of viral DNA replication, late transcription, and capsid assembly show a general overlapping distribution within replication compartments but appear to be located at distinct sites within these regions of the infected cell nucleus.

INTRODUCTION

Herpes simplex virus (HSV) is a double-stranded DNA virus that replicates in the host cell nucleus. Consequently, a variety of viral processes occur within the nucleus, including transcription, DNA synthesis and processing, and nucleocapsid assembly. These events take place within a relatively short period of time and are highly regulated. Viral gene expression is not only regulated in a cascade involving at least three distinct kinetic classes, immediate early (IE), early (E), and late (L), but also is temporally coordinated with viral DNA synthesis (reviewed in Knipe, 1996; Roizman and Sears, 1996). Much as analogous cellular activities are organized within the cell nucleus (reviewed in Strouboulis and Wolffe, 1992), efficient coordination of these viral functions may rely in part on the spatial organization of viral functions within the infected cell nucleus.

Several lines of evidence have already established that HSV DNA replication occurs within specific domains in the infected cell nucleus, large globular structures called replication compartments. The boundaries of these compartments were originally defined by immuno-

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those where transcription takes place (Pombo et al., 1994). First, in HSV-infected cells, ICP4, the major transcriptional activator, is found within replication compartments (Randall and Dinwoodie, 1986; Knipe et al., 1987; Leopardi et al., 1997). Second, the cellular RNA pol II, which is responsible for viral gene transcription (Alwine et al., 1974; Costanzo et al., 1977), is also localized to these domains (Rice et al., 1994; Leopardi et al., 1997). Finally, pulse-labeling of RNA in infected cells, believed to detect viral transcription, has shown that active transcription occurs in structures that overlap with replication compartments (Phelan et al., 1997).

Despite the fact that splicing factors are usually found associated with transcription sites in eukaryotic cells (Carmo-Fonseca et al., 1992; Jimenez-Garcia and Spectrum, 1993; Bregman et al., 1995), host cell splicing factors are excluded from HSV replication compartments (de Bruyn Kops, 1991; Phelan et al., 1997). This is due, at least in part, to the HSV ICP27 protein which is required for the viral-induced inhibition of splicing in infected cells and the apparent clumping of splicing factors into prominent clusters which migrate to the nuclear periphery as infection proceeds (Martin et al., 1987; Phelan et al., 1993; Sandri-Goldin et al., 1995; McGregor et al., 1996).

Some reports have indicated that ICP27 shows a diffuse distribution within infected and transfected cell nuclei (Knipe and Smith, 1986; Rice et al., 1989; Zhu et al., 1994; Zhu and Schaffer, 1995) while others have indicated that ICP27 colocalizes with cellular RNA splicing factors in spliceosomes in the infected cell nucleus (Phelan et al., 1993; Sandri-Goldin et al., 1995; Phelan and Clements, 1997).

Progeny viral DNA appears to remain within replication compartments following its synthesis (Rixon et al., 1983; de Bruyn Kops and Knipe, 1988; Knipe, 1990), and viral DNA replication, recombination, cleavage, and packaging are thought to be closely linked (Preston et al., 1983; Sherman and Bachenheimer, 1987; Weber et al., 1988; Desai et al., 1993; Gao et al., 1994; Matusick-Kumar et al., 1994). Thus it might be expected that cleavage and packaging of viral genomes also occur in or near replication compartments. Consistent with this hypothesis, several reports have contained immunofluorescence images suggesting that various HSV capsid proteins, such as VP5 (Church and Wilson, 1997), VP19c or UL38, (Chowdhury and Batterson, 1994), and p12 (McNabb and Courtney, 1992), partially localize to globular nuclear domains which appear identical to replication compartments. Other reports of nuclear structures distinct from replication compartments containing ICP5, VP19c, and ICP35 (Nalwanga et al., 1996; Ward et al., 1996) argued that there may be separate sites for assembly of capsids as the name for these structures, "assemblons," implied (Ward et al., 1996). Since the definition of assemblons has been reported, however, recent papers have indicated that the DNA-packaging proteins, UL6 (Phelan et al., 1997) and UL15 (Ward et al., 1996; Yu and Weller, 1998), and assembled HSV capsids (Lamberti and Weller, 1998) colocalize with replication compartments at various times during infection. Thus the spatial relationship between viral DNA replication and assembly sites remains controversial.

While it has been demonstrated that viral replication compartments extend through the nucleus and are assembled at locations determined by the host cell architecture (de Bruyn Kops and Knipe, 1994), i.e., adjacent to ND-10 structures (Ishov and Maul, 1996; Maul et al., 1996), it is also clear that these are dynamic structures which can form and change in response various cellular and viral factors. For example, when viral DNA synthesis is prevented, many of the replication proteins are found in smaller structures with a punctate distribution called prereplicative sites (Quinlan et al., 1984; Olivo et al., 1989; Bush et al., 1991; Goodrich et al., 1990; Liptak et al., 1996; Lukonis and Weller, 1996). ICP8 has been found to reversibly distribute between prereplicative sites and replication compartments depending on the status of viral DNA synthesis (Quinlan et al., 1984). Hence replication compartments are not static structures but active domains capable of adjusting to the changing needs of the virus during the productive infection cycle.

The presence of DNA replication proteins, transcriptional enzymes and activators, and viral structural proteins together within replication compartments suggests that these dynamic structures are multifunctional. The observation that there are foci of ICP8 staining within the replication compartments that correspond to sites of DNA synthesis (de Bruyn Kops and Knipe, 1988, 1994) further implies that replication compartments may contain subdomains in which these functions are compartmentalized. Nevertheless, as described above, there is considerable controversy in the literature regarding the intranuclear distributions of the different nuclear gene products and processes in HSV-infected cells. To further investigate the compartmentalization of viral functions within the infected cell nucleus, we have used double label immunofluorescence and confocal microscopy to investigate the organization of HSV proteins involved in various processes within the infected cell nucleus.

**RESULTS**

Previous studies as detailed above have shown that a number of HSV proteins involved in DNA synthesis, transcription, and capsid assembly are compartmentalized within the infected cell nucleus. To better understand the relative distributions of the viral proteins involved in these processes, we have utilized double label immunofluorescence and confocal microscopy to define more precisely the intranuclear distributions of several HSV proteins.
Time course of HSV-1 infection of CV-1 cells

We have chosen to use CV-1 cells for these immuno-fluorescence studies because these cells and their nuclei are relatively flat, allowing better resolution of intranuclear structure by light microscopy (de Bruyn Kops and Knipe, 1988). Because correlations between protein localization and function have to be made at the appropriate time during infection, we wished to examine infected cell nuclei at times when viral transcription, DNA synthesis, and capsid assembly were occurring simultaneously, and hence, when all classes of viral proteins involved in these processes are present. We therefore examined the time course of viral protein synthesis, viral DNA synthesis, and viral progeny production in CV-1 cells infected with KOS1.1 virus at an m.o.i. of 20 PFU/cell. Under these conditions, synthesis of viral proteins of all three kinetic classes, IE, E, and L, occurred from 3.5 to 5.5 h p.i. (Fig. 1). Viral DNA synthesis started between 3.5 and 5.5 h p.i. and continued through 18 h p.i. (Fig. 2A). Accumulation of infectious progeny virus also initiated prior to 5.5 h p.i. (Fig. 2B). By 6±0.5 h p.i., infected cells exhibited some effects of cytopathogenicity (results not shown). Therefore, we chose 5.5 h p.i. as the time for our initial studies to examine the intranuclear distribution of IE, E, and L viral proteins under conditions where there was minimal cytopathic effect.

Several viral DNA synthesis proteins localize to punctate foci within replication compartments

We had shown previously that the HSV ICP8 DNA-binding protein localizes to punctate sites within replication compartments and that these punctate sites colocalize with sites of BrdU incorporation or sites of viral DNA synthesis (de Bruyn Kops and Knipe, 1994). The HSV DNA polymerase (pol) had been shown to localize to replication compartments (Goodrich et al., 1990; Bush et al., 1991). To define the distribution of pol within replication compartments, we dual stained infected cells for pol and ICP8 and examined them by confocal microscopy. Optical sections from the top through the bottom of the nucleus demonstrated that ICP8 and pol were both concentrated at the same foci within the replication compartments (Fig. 3). The actual colocalization of ICP8 and pol at these punctate sites can be seen more definitively in color micrographs where the red pol staining and the green ICP8 staining overlapped to give a yellow punctate pattern within replication compartments (Fig. 4A).
Although not specifically addressed in previous reports, it is worth noting that immunofluorescence staining of the HSV helicase–primase proteins, UL5, UL8, and UL52, in infected cells also reveals colocalization of these proteins with ICP8 at punctate sites within replication compartments (Liptak et al., 1996; Lukonis and Weller, 1996). Thus at least five of the seven HSV DNA replication proteins are localized specifically with ICP8 at foci of DNA synthesis rather than being randomly distributed throughout replication compartments.

Two regulatory proteins, ICP4 and ICP27, are distributed differently from DNA replication proteins within replication compartments.

The HSV transcriptional activator, ICP4, localizes to replication compartments (Randall and Dinwoodie, 1986;
Knipe et al., 1987). To determine the subnuclear distribution of ICP4, we stained infected cells with the anti-ICP8 3–83 rabbit serum and the anti-ICP4 58S MAB. ICP4 localization to replication compartments was observed as early as 3.5 h p.i. and remained in this distribution through 24 h p.i. (data not shown). At 5.5 h p.i., optical sections showed that ICP4 did not show a punctate pattern (Fig. 5). Instead, the ICP4 distribution appeared to be more uniform with somewhat higher intensity of staining near the center of the compartments. Furthermore in contrast to the coincidence of ICP8 and pol staining images (Fig. 4A), color micrographs showed a punctate pattern of ICP8 staining over a diffuse pattern of ICP4 staining (Fig. 4B).

Previous studies on the intracellular localization of the ICP27 regulatory protein have reported either a diffuse nuclear distribution for the ICP27 protein (Knipe and Smith, 1986; Rice et al., 1989; Zhu et al., 1994; Zhu and Schaffer, 1995) or a codistribution with spliceosomes (Phelan et al., 1993; Sandri-Goldin et al., 1995). To define the intranuclear location of ICP27 more precisely, we stained infected cells with the anti-ICP8 3–83 rabbit serum and the anti-ICP27 MAB H1113. Optical series of ICP27 staining showed that ICP27 exhibited staining of replication compartments as well as additional sites outside the boundaries of these compartments. In particular, ICP27 accumulated at the top and bottom of the nucleus, in areas not overlapping sites of ICP8 accumulation (Fig. 6). ICP27 staining within compartments was generally diffuse in appearance; however, there also was some granular concentrations of ICP27 within compartments, which may in part coincide with ICP8 foci. Thus although ICP4 and ICP27 showed somewhat different overall distributions, neither of these gene regulatory proteins displayed a punctate distribution coincident with ICP8 but rather showed a more general staining within replication compartments.

UL42 protein is distributed diffusely within replication compartments

The HSV UL42 protein is required for viral DNA synthesis and serves as a polymerase accessory protein in that it complexes with the catalytic UL30 pol subunit to increase its processivity (Gallo et al., 1989; Gottlieb et al., 1990; Hernandez and Lehman, 1990). UL42 localizes to replication compartments (Goodrich et al., 1990; de Bruyn Kops, 1991); however, to date no one has detected any accumulation of UL42 at prereplicative sites. To further define the intranuclear distribution of UL42 within replication compartments, we dual stained infected cells with the anti-UL42 R232 rabbit serum and the 39S anti-ICP8 mouse MAB. Unlike the other DNA replication proteins examined above, UL42 was distributed diffusely within the replication compartments (Fig. 7). A similar diffuse distribution for UL42 was observed when the 5H11D6 anti-UL42 mouse MAB was used (Fig. 4C; Fig. 8K). Similar to the image observed for ICP4, in dual color micrographs, UL42 showed a diffuse distribution superimposed on the punctate distribution of ICP8 (Fig. 4C).
FIG. 10. Intranuclear localization of ICP5 relative to ICP8. Infected CV-1 cells were processed for immunofluorescence at various times after infection. After acetone permeabilization, staining for ICP8 and ICP5 was performed. Panels are as follows: (A–C), 6 h p.i., (A), ICP8 detected by 10-E3 MAB; (B), ICP5 detected by NC1 serum; (C), merged image of (A) and (B); (D–F), 12 h p.i., (D), ICP8 detected by 10-E3 MAB; (E), ICP5 detected by NC1 serum; (F), merged image of (D) and (E); (G–I), 8 h p.i., (G), ICP8 detected by 3E83 serum; (H), ICP5 in hexons detected by 6F10 MAB; (I), merged image of (G) and (H); (J–L), 18 h p.i., (J), ICP8 detected by 3E83 serum; (K), ICP5 in hexons detected by 6F10 MAB; (L), merged image of (J) and (K).
The distribution patterns of viral proteins within replication compartments correlate with their behavior upon inhibition of viral DNA synthesis.

ICP8 redistributes between prereplicative sites and replication compartments depending on the status of viral DNA synthesis (Quinlan et al., 1984). Specifically, if viral DNA replication is inhibited, ICP8 redistributes from replication compartments to prereplicative sites. In contrast, ICP4 does not redistribute once viral DNA synthesis is inhibited but remains in structures resembling replication compartments (Knipe et al., 1987). These patterns were also observed in the presence of cycloheximide, demonstrating that they were due to preexisting proteins rather than newly synthesized polypeptides.

FIG. 5. Optical series comparing the three-dimensional distribution of ICP8 and ICP4. Infected CV-1 cells were processed for immunofluorescence at 5.5 h p.i. After Triton X-100 permeabilization, cells were stained with the 383 polyclonal antisera specific for ICP8 and the 58S monoclonal antibody specific for ICP4. The cells were then stained with FITC-conjugated goat anti-mouse and biotin-conjugated goat anti-rabbit secondary antibodies followed by Texas red-conjugated avidin. Optical sections were obtained as in Fig. 1. (A–F) Continuous optical series traversing the nucleus from bottom to top. The left image of each panel shows ICP8 staining, the right image shows ICP4 staining. Images were pseudocolor mapped as described in Fig. 1. Bar equals 5 μm.
To determine the behavior of other viral proteins and newly synthesized DNA within replication compartments upon inhibition of viral DNA synthesis, we monitored the location of pol, BrdU-labeled DNA, ICP4, ICP27, and UL42 relative to ICP8 after blocking viral DNA synthesis.

First, cells were infected, and replication compartments were allowed to form. At 4 h p.i., one set of coverslips was fixed and stained to confirm the presence of replication compartments within infected cells, while PAA was added to the duplicate cultures followed by further incubation at 37°C for 4±5 h before being processed for immunofluorescence. By 4 h p.i., ICP8 (Fig. 8A), pol (Fig. 8C), BrdU-labeled DNA (Fig. 8E), ICP4 (Fig. 8G), ICP27 (Fig. 8I), and UL42 (Fig. 8K) were all localized to replication compartments as described above. However, after an additional incubation period with PAA to inhibit viral DNA synthesis, ICP8 (Fig. 8B), pol (Fig. 8D), and BrdU-labeled DNA (Fig. 8H) had redistributed from replication compartments. After inhibition of viral DNA synthesis, pol (Fig. 8D) displayed a prereplicative site pattern coincident with ICP8 (Fig. 8B), while labeled DNA, which could represent viral or cellular DNA, was redistributed throughout the nucleus (Fig. 8F). The other proteins studied did not redistribute in response to the cessation of

FIG. 6. Optical series comparing the three-dimensional distributions of ICP8 and ICP27. Infected CV-1 cells were processed for immunofluorescence at 5.5 h p.i. After Triton X-100 permeabilization, cells were stained with the 3–83 polyclonal antiserum specific for ICP8 and the H1113 monoclonal antibody specific for ICP27. Cells were then stained with FITC-conjugated goat anti-mouse and biotin-conjugated goat anti-rabbit secondary antibodies followed by Texas red-conjugated avidin. (A–F) Continuous optical series traversing the nucleus from bottom to top. The left image of each panel shows ICP8 staining, and the right image shows ICP27 staining. Images were pseudocolor mapped as described in Fig. 1. Bar equals 5 µm.
viral DNA synthesis. After addition of PAA, ICP4 (Fig. 8H), ICP27 (Fig. 8J), and UL42 (Fig. 8L) were still primarily associated with replication compartments, clearly distinct from the punctate staining observed for ICP8 in these same cells (results not shown). Thus the viral proteins that localized to punctate foci within replication compartments redistributed from replication compartments to prereplicative sites following inhibition of viral DNA synthesis, whereas the viral proteins showing a diffuse distribution in the replication compartments, ICP4, ICP27, and UL42, did not move from the compartments.

The major capsid protein, ICP5, initially localizes to a diffuse pattern in replication compartments but then moves to distinct punctate foci within compartments. Structures distinct from replication compartments have been hypothesized to be the sites of capsid assembly, and these structures have been called "assemblons."
(Ward et al., 1996); however, others have recently observed some DNA packaging/cleavage proteins and a capsid protein in replication compartments (Phelan et al., 1997; Lamberti and Weller, 1998; Yu and Weller, 1998). To determine the intranuclear distribution of ICP5, we dual stained infected cells at 5.5 h p.i. with the NC1 anti-ICP5 rabbit serum (Cohen et al., 1980) and the 39S anti-ICP8 MAB. Optical series of ICP5-stained cells showed that this protein was localized within replication compartments at 5.5 h p.i. (Fig. 9). In some optical sections, ICP5 staining appeared strongest in areas of the compartments where ICP8 staining was fainter.

To monitor the progressive movement of ICP5 within the nucleus in attempt to define the sites of nucleocapsid assembly, we then examined the distribution of ICP5 as a function of time following infection using two ICP5 antibodies, the NC1 anti-ICP5 rabbit serum, which likely recognizes both unassembled and assembled forms of ICP5 (Lee and Knipe, 1983), and the 6F10 MAB, which recognizes ICP5 assembled in hexons (Newcomb et al., 1996). Using the NC1 serum, we observed diffuse nuclear staining with some concentration of ICP5 in and near replication compartments at early times (3.5–6 h p.i.) (Fig. 10B). At later times (8–24 h p.i.), NC1 staining showed additional foci of ICP5 in punctate structures (Fig. 10E) that localized primarily within replication compartments but not necessarily overlapping the punctate structures containing ICP8 (Fig. 10F). Using the 6F10 MAB, we observed assembled ICP5 in a few punctate structures in the nucleus by 5.5±8 h p.i. (Fig. 10H), again mostly within replication compartments (Fig. 10I). There were a few punctate sites outside of replication compartments that were labeled with 6F10, but these were typically adjacent to a punctate site of ICP8 staining (Fig. 10I). At late times, numerous foci stained with 6F10 (Fig. 10K) were observed in replication compartments adjacent to and intermixed with ICP8 sites (Fig. 10L). Thus ICP5 showed a diffuse distribution within the nucleus and replication compartments at early times p.i. This likely represented ICP5 not assembled into hexons because the hexon-specific MAB recognized only a few punctate sites in these nuclei. ICP5 reactive with 6F10 and thus assembled in hexons accumulated at punctate sites within replication compartments throughout the time of maximal progeny virus assembly, 5.5±18 h p.i. (Fig. 2). We conclude that during this period, ICP5 in hexons showed a distribution different from those of the DNA replication proteins and the viral regulatory proteins. Therefore it is likely that capsid assembly and/or encapsidation of DNA occur within a subcompartment of replication compartments that is different from the sub-

anti-polymerase MAB, Be5; (E and F), anti-BrdU MAB; (G and H), anti-ICP4 MAB, 58S; (I and J), anti-ICP27 MAB, H1113; (K and L), anti-UL42 MAB, 5H11D6.

FIG. 8. Localization of replication compartment proteins before and after inhibition of viral DNA synthesis. CV-1 cells were infected as described. Some cells were labeled with BrdU from 3.25 to 3.75 h p.i. One set of infected coverslips was fixed and processed for immunofluorescence with acetone permeabilization at 4 h p.i. (left column). In parallel infections viral DNA synthesis was blocked by incubating infected cells with 400 μg/ml PAA for an additional 4±5 h before immunofluorescence staining (right column). Cells were dual stained for ICP8 with 3±83 serum (A and B and data not shown) and for the following proteins with the indicated antibodies: (C and D), (Ward et al., 1996); however, others have recently observed some DNA packaging/cleavage proteins and a capsid protein in replication compartments (Phelan et al., 1997; Lamberti and Weller, 1998; Yu and Weller, 1998). To determine the intranuclear distribution of ICP5, we dual stained infected cells at 5.5 h p.i. with the NC1 anti-ICP5 rabbit serum (Cohen et al., 1980) and the 39S anti-ICP8 MAB. Optical series of ICP5-stained cells showed that this protein was localized within replication compartments at 5.5 h p.i. (Fig. 9). In some optical sections, ICP5 staining appeared strongest in areas of the compartments where ICP8 staining was fainter.

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compartments within which viral DNA synthesis and late transcription occur.

**DISCUSSION**

These experiments have shown that HSV DNA replication proteins, regulatory proteins, and the major capsid protein all localize to replication compartments at times when they are actively involved in DNA synthesis, viral transcription, or capsid assembly, but that they show distinct distributions within these structures. In total, these results argue that these various viral processes are subcompartmentalized within the larger replication compartment structures. We will examine each of these subcompartments in turn.

**FIG. 9.** Optical series comparing the three-dimensional distribution of ICP8 and ICP5. Infected CV-1 cells were processed for immunofluorescence at 5.5 h p.i. After Triton X-100 permeabilization, cells were stained with the 39S monoclonal antibody specific for ICP8 and the NC1 polyclonal antiserum recognizing ICP5. The cells were then stained with FITC-conjugated goat anti-rabbit and biotin-conjugated goat anti-mouse secondary antibodies followed by Texas red-conjugated avidin. Series of dual channel optical sections were collected as in Fig. 3. (A–F) Continuous optical series traversing the nucleus from bottom to top. The left image of each panel shows ICP8 staining, and the right image shows ICP5 staining. Images were pseudocolor mapped as described in Fig. 1. Bar equals 5 μm.
Viral DNA synthesis subcompartment

We had shown previously that the ICP8 DNA-binding protein and the sites of BrdU incorporation were colocalized at punctate structures within the replication compartments (de Bruyn Kops and Knipe, 1994), providing the first evidence for this subcompartment. This study and previous studies (Liptak et al., 1996; Lukonis and Weller, 1996) have shown that four additional replication proteins, UL5, UL8, UL52, and pol, also colocalize at punctate sites within replication compartments. The punctate sites likely represent the sites of viral DNA synthesis as well as possibly cellular DNA synthesis. At least two of these proteins relocalize to a dispersed punctate distribution when viral DNA synthesis is inhibited (Quinlan et al., 1984; Knipe et al., 1987; these studies). Therefore association of the replication complexes and even newly synthesized DNA within this subcompartment may be dependent on ongoing DNA synthesis. Alternatively, functional pol may be necessary for association of the replication proteins with replication compartments. Further studies are necessary to define the molecular basis for this interesting but surprising dependence on DNA synthesis or pol for compartment localization.

In contrast, the seventh viral DNA replication protein, UL42, is not detected at prereplicative sites, does not show a punctate distribution within replication compartments, and does not redistribute from replication compartments upon inhibition of viral DNA synthesis. It is likely that the UL42 protein is located within replication complexes, and it has been shown to promote the localization of HSV pol to prereplicative sites (Liptak et al., 1996). The lack of detection of UL42 in prereplicative sites could be due to masking of the protein within the replication complexes (Liptak et al., 1996). Meanwhile, the diffuse distribution of UL42 in replication compartments could reflect a secondary activity of the UL42 molecule that targets a large portion of the protein to other subcompartments within this structure.

Regulatory protein subcompartment

The regulatory proteins ICP4 and ICP27 show a more diffuse distribution within replication compartments compared to ICP8 and pol. This difference alone would not be sufficient to identify this association as different from that of the DNA synthesis proteins. However, both of these proteins also remain in replication compartment structures when viral DNA synthesis is inhibited, providing further evidence that their association with replication compartments is distinct from that of the DNA replication proteins. ICP4 acts as a transcriptional activator of E and L gene expression, possibly by interacting with cellular transcription factors (Papavassiliou and Silverstein, 1990; Papavassiliou et al., 1991; Smith et al., 1993; Gu and DeLuca, 1994), and its presence in replication compartments is thought to reflect its role in stimulating L gene expression (Knipe et al., 1987; Rice et al., 1994; Leopardi et al., 1997). Thus this diffuse distribution of ICP4 may mark a sub-compartment of the replication compartment where L gene transcription is taking place. Although others have reported that viral replication sites colocalized with transcription sites at early times of infection but are present at larger numbers than transcription sites at late times (Phelan et al., 1997), we have observed codistribution of ICP8 and ICP4 through 24 h p.i. Therefore under our conditions of infection, replication and transcription sites appear to overlap within the boundaries of replication compartments through late times after infection.

Numerous functions or effects of ICP27 have been reported, including stimulation of late gene expression (Sacks et al., 1985; McCarthy et al., 1989; Rice et al., 1989), inhibition of host cell RNA splicing (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994), stimulation of early gene expression (McGregor et al., 1996; Samaniego et al., 1995; Uprichard and Knipe, 1996), shuttling of RNAs to the cytoplasm (Phelan and Clements, 1997; Soliman et al., 1997; Mears and Rice, 1998), regulation of differential polyadenylation (McLauchlan et al., 1992; Hann et al., 1998), and stimulation of transcription of at least certain late viral genes (Jean et al., unpublished observations). Furthermore, the intranuclear distribution of ICP27 has been controversial in that some have reported diffuse intranuclear distributions for ICP27 in infected and transfected cells (Knipe and Smith, 1986; Rice et al., 1989; Zhu et al., 1994; Zhu and Schaffer, 1995) while other studies have reported that ICP27 shows a more speckled appearance and colocalizes with snRNPs in the infected cell nucleus (Phelan et al., 1993; Sandri-Goldin et al., 1995; Phelan and Clements, 1997). The reason(s) for the observed discrepancies in ICP27 localization is unknown; however, differences in the anti-ICP27 antibody used for detection may explain the distributions observed. The studies observing ICP27 colocalizing with snRNPs have used anti-peptide rabbit sera (Phelan et al., 1993) or other rabbit sera (Sandri-Goldin et al., 1995) while the studies showing ICP27 in a diffuse distribution or within replication compartments have used a monoclonal antibody (Knipe and Smith, 1986; Rice et al., 1989; Zhu and Schaffer, 1995). Thus the various reagents may recognize different forms of ICP27 or show different degrees of specificity.

Using H1113 MAB and confocal microscopy, this study found ICP27 to be localized to a significant extent within replication compartments and at the top and bottom of the cell nucleus. Presumably, visualization of the ICP27 distribution by conventional microscopy in our previous studies showed an apparent diffuse distribution due to the superimposition of these different distributions of ICP27. ICP27 has also been reported to interact with ICP4 (Panagiotidis et al., 1997), and this could play a role in targeting of ICP27 to replication compartments. Be-
cause it is not possible to define the precise mechanisms of action of ICP27 at this time, it is not yet feasible to correlate ICP27 function with intracellular location in any rigorous way. However, it is tempting to hypothesize that the population of ICP27 molecules localizing to replication compartments reflects its potential role in interacting with ICP4 and in some way promoting transcription and/or transport of transcripts from the site of transcription while the population of ICP27 localizing at the top and bottom of the nucleus reflects ICP27 shuttling to and from the nucleus.

Capsid protein subcompartment

Several observations in the literature of various HSV capsid proteins localizing to globular nuclear domains similar in appearance to replication compartments (McNabb and Courtney, 1992; Chowdhury and Batterson, 1994; Church and Wilson, 1997) suggested that capsid proteins assemble into capsids and/or encapsidate progeny viral DNA in those nuclear compartments. However, the description of nuclear structures distinct from replication compartments containing ICP5, VP19c and ICP35 (Nalwanga et al., 1996; Ward et al., 1996) led those authors to hypothesize that viral DNA molecules synthesized in the "DNA synthesis compartment" are encapsidated in "assemblon" structures at the periphery of replication compartments. In contrast, other dual label immunofluorescence studies showed that assembled ICP5 colocalizes with replication compartments at 6 and 8 h p.i. (Lamberti and Weller, 1998). We have observed that ICP5 localizes first to a diffuse distribution both within the nucleus as well as specifically within replication compartments. Later, capsid-assembled ICP5 localizes to punctate structures primarily within replication compartments. Although foci of ICP5 staining were observed occasionally outside replication compartments, they were usually adjacent to sites of ICP8 staining. Hence our results argue that assembled ICP5 accumulates in punctate sites within replication compartments. Capsid assembly could occur at those sites or at other locations within replication compartments followed by quick movement to these punctate sites. Because the sites of assembled ICP5 accumulation are generally adjacent to DNA synthesis sites during the times of maximal production of progeny virus, 5.5±0.8 h p.i., we favor the hypothesis that viral DNA is encapsidated in capsid protein subcompartments within replication compartments.

At this time we do not have a complete explanation for the apparent differences in results and interpretation regarding intranuclear capsid assembly sites. Ward et al. (1996) reported studies of infected cells only at late times, 16±2 h p.i., and thus the "assemblons" they described may be, as also suggested by Lamberti and Weller (1998), late, dead-end products rather than assembly intermediates. We do feel that it is important to study protein localization at the appropriate times during infection; thus we have studied capsid protein localization throughout infection and specifically during the period of maximal virus production. At these times, the bulk of ICP5 localized within replication compartments and not to "assemblons."

This study shows that the processes of DNA synthesis, late gene transcription, and nucleocapsid assembly are likely to be occurring in overlapping functional subcompartments or domains within the replication compartments. The physical proximity of these processes may allow a functional and physical linkage between these related steps in viral replication. The distinct intranuclear distributions of the proteins involved in each of these steps argue that the viral DNA molecule is targeted to a unique set of molecular sites in the nucleus for each of these stages of the viral life cycle. Most importantly, these studies point to the need for a molecular definition of each of the interactions of the viral DNA molecule with cellular and viral proteins and the cell nucleus as it is transcribed, replicated, and packaged into capsids within the infected cell nucleus.

MATERIALS AND METHODS

Cells and virus strains

African green monkey kidney cells (CV-1; American Type Culture Collection, Rockville, MD) were used for all experiments. Cells were grown in Dulbecco's modified Eagles medium (DME, Irvine Scientific, Santa Ana, CA) containing 10% heat-inactivated fetal calf serum (Gibco/BRL, Gaithersburg, MD), L-glutamine (2 mM, Flow Laboratories), 0.0242% streptomycin sulfate (Sigma Chemicals, St. Louis, MO) and 2.4 × 10^2 units/ml penicillin G potassium (Bristol-Myers Squibb, Princeton, NJ). All experiments involved infection of CV-1 cells with HSV-1 strain KOS 11 wt virus (Hughes and Munyon, 1975) at a m.o.i. of 20 as titrated on CV-1 cells. Virus was diluted in cold phosphate-buffered saline (PBS) containing 0.1% glucose and 10% heat-inactivated newborn calf serum (Gibco/BRL) and incubated with CV-1 cells for 1 h at 37°C. The overlay medium was changed to 199 medium-1% heat-inactivated calf serum.

Metabolic labeling of viral proteins

CV-1 cells were infected with wt HSV as described above. At the times indicated, infected cultures were pulse-labeled for 15 min with 100 µCi/ml [35S]methionine of specific activity 1186 Ci/mmol (ICN Biomedicals, Costa Mesa, CA) in methionine-free minimal essential medium (ICN) containing 1% FCS. Pulse-labeling was initiated and terminated quickly by submerging flasks in a 37°C water bath and 0°C ice bath, respectively, as described previously (Knipe and Spang, 1982). Cells were harvested immediately following the pulse label in ice-cold...
PBS containing 100 mM phenylmethanesulfonyl fluoride (PMSF) and 2 mM Na-p-tosyl-L-lysine chloro-methyl Ketone (TLCK) and collected by centrifugation. Cell pellets were resuspended in SD5 sample buffer and proteins resolved by SD5 PAGE in a 9.25% polyacrylamide gel (Knipe and Spang, 1982). Following electrophoresis, gels were fixed, dried, and exposed to Kodak BioMax x-ray film for autoradiographic imaging.

**Measurement of virus yield and viral DNA level**

Infected cell cultures were harvested at the times indicated to determine virus yield and viral DNA level. Virus yield was measured by plaque assay titration of total intracellular and extracellular virus on CV-1 cells. Viral DNA was quantified using a hybridization protocol described previously (Rice and Knipe, 1990). Briefly, DNA was isolated from infected cell cultures, and fivefold serial dilutions of each DNA sample were immobilized onto a nylon membrane using a slot–blot apparatus (Schleicher and Schuell, Keene, NH). The filter was hybridized with $^{32}$P-labeled pSG28 plasmid DNA which contains the EcoRI EK fragment of the HSV-1 genome (Goldin et al., 1981). The intensity of the hybridized signal was quantified by phosphorimager analysis (Molecular Dynamics).

**Antibodies and reagents**

The 3–83 rabbit antiserum against ICP8 was described previously (Knipe et al., 1987) and was used at a 1:50 dilution. The ICP8 MAB, 39S (Showalter et al., 1981), was prepared from ascites fluid of animals inoculated with hybridoma cells derived from cultures originally obtained from ATCC and was used at a 1:25 dilution. The ICP8 MAB, 10E3 (Rose et al., 1986), obtained from Kathleen Shriver was used at a 1:40 dilution. The ICP4 MAB, 58S, (Showalter et al., 1981) was a gift from Neal DeLuca, Univ. of Pittsburgh, and was used at a 1:100 dilution. The H1113 MAB recognizing ICP27 (Ackermann et al., 1984) was purchased from the Goodwin Institute (Plantation, FL) and used at a dilution of 1:100. The NC1 rabbit serum specific for ICP5 (Cohen et al., 1980) was provided by R. Eisenberg and G. Cohen and was used at a dilution of 1:100. The 6F10 MAB specific for ICP5 in hexons of capsids and procapsids (Newcomb et al., 1996; Spencer et al., 1997) was provided by Jay Brown and was used at a dilution of 1:200. The R232 antiserum specific for UL42 (Olivo et al., 1989) was a gift from P. Olivo and M. Chalberg and was used at a dilution of 1:10. The 5H11D6 MAB (Gao et al., 1993) specific for UL42 was a gift from M. Gao and R. Colonna and used at a dilution of 1:30. The PP5 antiserum (Yager et al., 1990) and Be5 MAB (Weisshart et al., 1991) specific for the HSV polymerase (used at dilutions of 1:20 and 1:10 respectively) were both received from D. Coen. The anti-BrdU monoclonal antibody (1:10 dilution) was purchased from Becton Dickin-

son. RITC-conjugated goat anti-mouse (1:100 dilution), FITC-conjugated goat anti-mouse (1:100 dilution), biotin-conjugated goat anti-mouse (1:100), FITC-conjugated goat anti-rabbit (1:200), and biotin-conjugated goat anti-rabbit (1:100) immunoglobulin antibodies and Texas red conjugated avidin were all purchased from Cappel Laboratories.

**Indirect immunofluorescence microscopy**

Cells were grown on glass coverslips and infected with virus. When indicated, the viral-specific polymerase inhibitor sodium phosphonoacetate (PAA; Sigma) was added to the overlay media of infections at 4 h p.i. to a concentration of 400 μg/ml to stop viral DNA replication (Mao et al., 1975; Leinbach et al., 1976). DNA synthesis was detected in situ by labeling cells with the thymidine analog bromodeoxyuridine (BrdU; Sigma). Infected cells were labeled with 50 μM BrdU for 30 min from 3.25 to 3.75 h p.i., rinsed thoroughly, and chased in fresh media until fixation at the indicated times. To expose the incorporated BrdU residues prior to immunofluorescence staining, BrdU-labeled cells were treated with 4 M HCl for 10 min after permeabilization. All cells were fixed for 10 min with 2% formaldehyde in PBS, pH 7.6. Coverslips were immediately rinsed with PBS and then with glass-distilled water prior to permeabilization. All samples processed for standard microscopy were incubated with antibody for 30 min at 37°C in a humid chamber. After three 5-min washes in PBS, 10 μl of PBS containing appropriate dilutions of the necessary primary antibodies were spread on each coverslip for dual antibody staining. The coverslips were incubated with antibody for 30 min at 37°C in a humid chamber. After three 5-min washes in PBS, 10 μl of PBS containing appropriate dilutions of goat secondary antibodies was added to each coverslip. The coverslips were again incubated at 37°C for 30 min in a humid chamber and washed three times for 5 min in PBS before being rinsed in glass-distilled water and mounted in glycerol gelatin (Sigma) containing 1.3 mg/ml p-phenyldiamine (Sigma) to retard bleaching.

All samples processed for standard microscopy were stained with rhodamine isothiocyanate (RITC)-conjugated anti-mouse and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibodies. All cells processed for confocal microscopy were stained with goat anti-mouse and anti-rabbit secondary antibodies conjugated to FITC and biotin. Cells were then reacted with Texas red-conjugated avidin. FITC and Texas red were used as fluorochromes because they give optimal separation of signal on the BioRad/MRC confocal system; however, the wavelength of the laser beam in this system excites the FITC fluorochrome better than the Texas red fluorochrome. The biotin/avidin staining sys-
system provided an additional amplification step that significantly improved the signal obtained with the Texas red fluorochrome. Staining reactions were designed so that ICP8 was always stained with the Texas red fluorochrome, but the biotin/avidin amplification did not alter the ICP8 staining pattern compared with FITC-stained samples. In either case ICP8 localizes to replication compartments concentrating in bright foci within the domains (data not shown).

Microscopy and image analysis

Standard immunofluorescence and phase-contrast-microscopic analyses were performed with a Zeiss standard microscope utilizing Plan Neofluor 63× and 100× objective lenses as described previously (Knipe et al., 1987). Dual image color micrographs were obtained by consecutive exposures with fluorescein and rhodamine filters (Knipe et al., 1987). Confocal immunofluorescence microscopy was done as described previously (de Bruyn Kops and Knipe, 1994) using a Bio-Rad/MRC-600 confocal imaging system mounted on a Zeiss Axiophot microscope equipped with a 63× Plan Apochromat objective lens. Optical series of infected cells were collected using the single and dual channel functions at stage motor increments of 0.4 μm. Scans were collected with the channel apertures closed. This setting gave an estimated focal depth of ~0.5±0.6 μm used in combination with a high numerical aperture objective (manufacturer’s estimate). Series were collected beginning several sections above the nucleus and continuing several sections past the nucleus. All sections showing significant staining were included in the final series.

Sixteen-bit images were collected and scaled by a factor of 5±10 before storage as 8 bit images with pixel intensities ranging from 0 to 255 to retain low intensity information. All images within a series were scaled by the same factor to preserve the relative fluorescence between bright and dim sections. Scale factors were selected such that the brightest intensities were <255. Images were displayed using a pseudocolor routine which maps different colors to different pixel intensity ranges. The pseudocolor scale used assigns red, pink, and white in the order of increasing intensity. When photographed on black-and-white film, this scale gives color ranges of black, gray, and white in the order of increasing intensity.

ACKNOWLEDGMENTS

We thank our colleagues for generously providing antibodies and antisera: Kathleen Shriver, Paul Olivo and Mark Chalberg, Don Coen, Roz Eisenberg and Gary Cohen, Jay Brown, Neal DeLuca, M. Gao, and Richard Colombo. This study was supported by Public Health Service Research Grants AI-20530 and CA-26345. A.D.B.K. was supported by an NSF predoctoral fellowship, and S.L.U. was supported by Public Health Service Training Grant Al-07245. Confocal microscopy was performed in the Harvard Medical School facility, which was supported by Public Health Service Grant S10RR04951.

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