Localization of the Herpes Simplex Virus Type 1 65-Kilodalton DNA-Binding Protein and DNA Polymerase in the Presence and Absence of Viral DNA Synthesis

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Using indirect immunofluorescence, well-characterized monoclonal and polyclonal antibodies, and temperature-sensitive (*ts*) mutants of herpes simplex virus type 1, we demonstrated that the 65-kilodalton DNA-binding protein (65K_{DBP}), the major DNA-binding protein (infected cell polypeptide 8 [ICP8]), and the viral DNA polymerase (Pol) colocalize to replication compartments in the nuclei of infected cells under conditions which permit viral DNA synthesis. When viral DNA synthesis was blocked by incubation of the wild-type virus with phosphonoacetic acid, the 65K_{DBP}, Pol, and ICP8 failed to localize to replication compartments. Instead, ICP8 accumulated nearly exclusively to prereplication sites, while the 65K_{DBP} was only diffusely localized within the nuclei. Although some of the Pol accumulated in prereplication sites occupied by ICP8 in the presence of phosphonoacetic acid, a significant amount of Pol also was distributed throughout the nuclei. Examination by double-labeling immunofluorescence of DNA⁻ *ts* mutant virus-infected cells revealed that the 65K_{DBP} also did not colocalize with ICP8 to prereplication sites at temperatures nonpermissive for virus replication. These results are in disagreement with the hypothesis that ICP8 is the major organizational protein responsible for attracting other replication proteins to prereplication sites in preparation for viral DNA synthesis (A. de Bruyn Kops and D. M. Knipe, *Cell* 55:857–868, 1988), and they suggest that other viral proteins, perhaps in addition to ICP8, or replication fork progression per se are required to organize the 65K_{DBP}.

Of the 72 proteins predicted to be encoded by the genome of herpes simplex virus type 1 (HSV-1) (21), only 7 have been shown to be directly involved in the replication of viral DNA. Wu and co-workers (40) showed by transfection experiments that these seven genes were required in *trans* for the replication of plasmids containing an HSV-1 origin of replication. That these same genes are involved directly in the synthesis of viral DNA during the virus replication cycle is consistent with genetic studies which initially demonstrated that each of these genes is essential for the production of viral DNA and infectious progeny virus (1, 9, 11, 19, 35, 39).

The proteins encoded by the seven replication genes have now been identified and include an origin-binding protein (UL9 [24]), three components of a helicase-primase complex (UL5, UL8, and UL52 [4]), a DNA polymerase (*pol* [11, 31]), a single-stranded DNA-binding protein (infected cell polypeptide 8 [ICP8] [30, 39]), and a 65-kilodalton DNA-binding protein (65K_{DBP}, UL42 [20, 27]). Several lines of evidence suggest that at least some of these proteins exist as a complex. Immunoaffinity columns charged with monoclonal antibody (MAb) to the 65K_{DBP} (8) or its HSV-2 equivalent protein, infected cell-specific polypeptide ICSP34,35 (38), bound not only the relevant reactive protein, but DNA

polymerase (Pol) and other replication proteins. These results suggested that binding of the immunologically unrelated proteins to the column occurred by virtue of protein-protein interactions with the immunoreactive protein. Indirect immunofluorescence studies of the intracellular localization of ICP8 (32), Pol (D. Knipe, personal communication), and Pol together with its associated proteins (33) also support the existence of a replication complex in that these proteins localized to intranuclear sites termed replication compartments (32).

Recently, we demonstrated that the 65K_{DBP} was capable of stimulating the activity of Pol and appeared to have the properties of a Pol accessory factor (7). Thus, we were interested in determining whether or not the 65K_{DBP} was found in the same replication compartments as ICP8 and Pol and whether its localization was dependent on the synthesis of viral DNA. Olivo and co-workers (25), using single-fluorochrome indirect immunofluorescence with well-defined antibodies, found that Pol, ICP8, and the 65K_{DBP} as well as UL9 localized to similar compartmentlike structures. It was not established, however, whether these proteins actually colocalized to these structures within the same cells. In this report, we used indirect immunofluorescence in double-fluorochrome labeling experiments to extend these findings and determined that the 65K_{DBP} colocalizes to replication compartments together with ICP8 and Pol during wild-type (wt) virus replication. Analysis of localization patterns of the 65K_{DBP} in cells infected with temperature-sensitive (*ts*) mutants defective in DNA replication genes or

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with wt virus in the presence of phosphonoacetic acid (PAA, a specific inhibitor of the HSV-1 Pol) demonstrated that active viral DNA synthesis is required for 65K_{DBP} localization to and maintenance within replication compartments. However, in the absence of viral DNA synthesis, the 65K_{DBP} failed to localize to the prereplicative sites in which ICP8 was found to accumulate. By contrast, the Pol colocalized with ICP8 to prereplication sites, although some remained diffusely localized within the nuclei of infected cells incubated in the presence of PAA.

MATERIALS AND METHODS

Production of cells and viruses. Vero cells were cultivated in Dulbecco minimum essential medium supplemented with 5% newborn calf serum and 2.5% fetal bovine serum. Stocks of HSV-1 wt strain KOS (37) and *ts* mutants were prepared by low-multiplicity passage in Vero cells as previously described (26). The following *ts* mutants were used in these studies and were derived from the KOS strain: *ts*J12, a mutant defective in the gene for glycoprotein B (16, 34); *ts*A16 and *ts*A24, defective in the gene for ICP8 (34, 39); *ts*D9, defective in the gene for Pol (3, 11, 31); and *ts*701, defective in the gene for the 65K_{DBP} (2, 19). The permissive temperature (pT) and nonpermissive temperature (npT) were 34 and 39.7°C, respectively.

Antibodies. The mouse MAb 6898 has been shown to be specific for the 65K_{DBP} (27) and was kindly provided by Ann Cross and Howard Marsden (Institute of Virology, Glasgow, Scotland). The mouse MAb 39S, specific for ICP8 (36), was a gift from Martin Zweig (National Institutes of Health, Bethesda, Md.). Both MAbs were derived from ascites fluid. Paul Olivo and Mark Challberg (National Institutes of Health) kindly provided the rabbit polyclonal antibody R219 as purified immunoglobulin G (IgG), directed to a β -galactosidase/ICP8 fusion protein. The Pol-specific antibody was raised in rabbits against a bacteriophage T7 gene 10/HSV-1 Pol fusion protein expressed in and partially purified from *Escherichia coli* (6). The IgG fraction was purified by DEAE-Tris Acryl (IBF Biotechnics, Inc., Salvage, Md.) chromatography. Both the anti-ICP8 and anti-Pol rabbit IgG fractions were absorbed with extracts of acetone-methanol (1:1)-fixed uninfected Vero cells before use in indirect immunofluorescence assays.

Immunofluorescence. Vero cells on glass tissue culture chamber slides (Miles Laboratories, Naperville, Ill.) were infected at a multiplicity of infection (MOI) of 20 PFU per cell. Adsorption was allowed to proceed at 37°C for 30 min, followed by incubation as described for each experiment. Cells were fixed in 3.7% formaldehyde and permeabilized for 2 min with -20°C acetone as described previously (10). In single-fluorochrome labeling experiments, the MAb or monospecific antibody was first added, the cells were washed with phosphate-buffered saline, and the secondary species-specific IgG (Cappel Laboratories, West Chester, Pa.) conjugated to either fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (RITC) was added (10). In double-labeling experiments, a second set of primary antibody-secondary antibody incubations was performed before the addition of mounting medium (90% glycerol, 10% phosphate-buffered saline, 150 mM propyl gallate [Eastman Kodak Co., Rochester, N.Y.]). In such experiments, each primary antibody was derived from a different animal species to prevent cross-reactivity.

Stained slides were viewed with a Zeiss fluorescence microscope with a 40 $\times$ or 100 $\times$ Neofluar objective. For

detection of fluorescence from FITC-conjugated antibodies, a filter set (green filter) consisting of an exciter filter for light with wavelengths of 455 to 490 nm, a beam splitter for less than 510 nm, and a barrier filter for greater than 520 nm (single-label experiments) or 520 to 560 nm (double-label experiments) was used. For detection of RITC fluorescence, a filter set (red filter) consisting of an exciter filter for 546 nm, a beam splitter for greater than 580 nm, and a barrier filter for greater than 590 nm was used. Control experiments revealed complete exclusion of RITC fluorescence with the green filter set and of FITC fluorescence with the red filter set (Fig. 1A to D). Photographs were taken with Kodak Tri-X or T-Max pan film for up to 2-min exposure times.

RESULTS

Colocalization of the 65K_{DBP} and ICP8 during productive infection. In a previous report (10), MAb 6898 was used to demonstrate that the 65K_{DBP} accumulates in nuclei of cells infected with HSV-1 strain KOS in a pattern similar to that characteristic of ICP8 accumulation. The large intranuclear structures containing ICP8 have been called replication compartments and are believed to be sites of active viral DNA synthesis (32). To demonstrate more conclusively that the 65K_{DBP} was concentrated in these replication compartments, we performed double-labeling immunofluorescence assays to detect ICP8 and the 65K_{DBP} simultaneously in the same cells. Binding of the mouse MAb 6898 to the 65K_{DBP} was detected with RITC-conjugated goat anti-mouse IgG, while binding of a rabbit polyclonal antibody directed against a β -galactosidase/ICP8 fusion protein (R219) was detected with FITC-conjugated goat anti-rabbit IgG. By employing filter systems specific for the emission spectrum of each fluorochrome, it was possible to discriminate unambiguously between the two fluorochromes. Thus, when the R219 (ICP8) antibody and both secondary antibodies but no 6898 (65K_{DBP}) MAb were added and cells were viewed with the green filter, ICP8 could be detected as stained compartments within nuclei of wt virus-infected cells 6 h postinfection (p.i.) (Fig. 1A). However, no fluorescence was observed when the same field was viewed with the red filter (Fig. 1B). In the reciprocal experiment when 6898 (65K_{DBP}) MAb and both secondary antibodies but no R219 (ICP8) antibody were added to fixed infected cells, the 65K_{DBP} was detected as brightly stained intranuclear inclusions with the red filter (Fig. 1D), although no staining was observed in the same field viewed with the green filter (Fig. 1C). Mock-infected cells when stained similarly to detect ICP8 (Fig. 1E) or 65K_{DBP} (Fig. 1F) displayed no immunofluorescence with the green or red filter set, respectively. These controls confirmed that not only was there no species cross-reactivity of the fluorochrome-conjugated secondary antibodies, but each filter set detected only its cognate fluorochrome.

Fixed wt virus-infected cells also were stained with R219 and FITC-conjugated goat anti-rabbit IgG to detect ICP8 and with MAb 6898 and RITC-conjugated goat anti-mouse IgG to detect 65K_{DBP}. When cells were viewed with the green filter to specifically detect those areas containing ICP8 (Fig. 1G), stained areas resembled the replication compartments reported by Quinlan and co-workers (32). The same field when viewed with the red filter to specifically detect 65K_{DBP}-containing areas revealed an identical pattern of localization (Fig. 1H). More extensive comparison of the infected monolayer revealed that all cells that displayed positive fluorescence exhibited colocalization of the 65K_{DBP} and ICP8 within intranuclear replication compartments.

FIG. 1. Colocalization of the 65K_{DBP} and ICP8. Vero cells mock infected (E and F) or infected with HSV-1 (KOS) at an MOI of 20 PFU per cell (A, B, C, D, G, and H) were fixed at 6 h p.i. as described in Materials and Methods. Cells were viewed with the green filter set to detect ICP8 (A, C, E, and G) and with the red filter set to detect the 65K_{DBP} (B, D, F, and H). (A and B [same field]) Infected cells were stained with ICP8 antibody R219, FITC-conjugated goat anti-rabbit IgG, and RITC-conjugated goat anti-mouse IgG and photographed with the green and red filters, respectively. (C and D [same field]) Infected cells were stained with FITC-conjugated goat anti-rabbit IgG, 65K_{DBP} antibody 6898, and RITC-conjugated secondary antibody and photographed with the green and red filters, respectively. (E) Mock-infected cells were stained with R219 and FITC-conjugated secondary antibody and viewed with the green filter. (F) Mock-infected cells were stained with MAb 6898 and RITC-conjugated secondary antibody and viewed with the red filter. (G and H [same field]) Infected cells were stained with R219, FITC-conjugated secondary antibody, MAb 6898, and RITC-conjugated secondary antibody and photographed with the green and red filters, respectively.

FIG. 2. Colocalization of Pol with 65K_{DBP} and ICP8. Vero cells were infected with wt virus (20 PFU per cell) and fixed at 6 h p.i. Cells were stained with the Pol antibody and FITC-conjugated goat anti-rabbit IgG and then with either 65K_{DBP} antibody 6898 (A and B) or ICP8 antibody 39S (C and D) and RITC-conjugated goat anti-mouse IgG. Cells in panels A and C were viewed with the green filter set to detect Pol, and the same fields (B and D, respectively) were viewed with the red filter set to detect 65K_{DBP} or ICP8.

Colocalization of the 65K_{DBP} and Pol. We used a rabbit polyclonal antibody raised against the HSV-1 DNA polymerase expressed in *E. coli* as a T7 gene 10/*pol* fusion under the control of a T7 RNA polymerase promoter and FITC-conjugated goat anti-rabbit IgG to detect Pol and MAb 6898 and RITC-conjugated goat anti-mouse IgG to detect 65K_{DBP} in double-labeling immunofluorescence to determine whether these proteins also colocalized in HSV-1-infected cells. In cells fixed at 6 h after infection with the wt virus and viewed with the green filter set, we detected Pol in intranuclear structures which resembled replication compartments (Fig. 2A). The same field viewed with the red filter set demonstrated that the 65K_{DBP} colocalized to the same compartments (Fig. 2B). In control slides stained individually with only one of the primary antibodies and both of the secondary antibodies as described above for Fig. 1, no cross-reactivity of secondary antibodies was detected, confirming that the filter set used excluded the wavelengths of light required to observe the other fluorochrome (data not shown). Furthermore, double-labeling immunofluorescence of cells fixed in parallel with the mouse MAb 39S and RITC-conjugated goat anti-mouse IgG to detect ICP8 and the Pol antibody with its FITC-conjugated secondary antibody demonstrated that with the green filter set (Fig. 2C), Pol localized to the same areas as the ICP8 detected with the red filter set (Fig. 2D). Thus, the 65K_{DBP}, Pol, and ICP8 all colocalize to replication compartments in wt virus-infected cells.

Localization of the 65K_{DBP}, Pol, and ICP8 in the presence of PAA. Because the 65K_{DBP} is required for origin-dependent

DNA replication (40) and present in replication compartments during productive infection, we next determined the effect of a chemical inhibitor of viral DNA synthesis, PAA, on the localization of the 65K_{DBP}. The drug PAA has been shown to specifically inhibit the activity of the viral Pol (17, 18). In the absence of PAA, both ICP8 and 65K_{DBP} colocalized in replication compartments at 7 h p.i. as demonstrated by the same pattern of staining observed when the field shown was viewed with the green (ICP8) or the red (65K_{DBP}) filter (Fig. 3A and B, respectively). However, in the presence of 400 μ g of PAA per ml, which is sufficient to completely block viral DNA synthesis (32; unpublished results), ICP8 (viewed with the green filter) localized to small discrete punctatelike areas (Fig. 3C) which have been termed prereplicative sites (32). In the same cells, the 65K_{DBP} (viewed with the red filter) was dispersed uniformly throughout the nucleus with the exception of the nucleoli (Fig. 3D), failing to localize to either the replication or prereplication compartments. These results suggested that active viral DNA replication was required for the 65K_{DBP} and ICP8 to localize to replication compartments. Moreover, they demonstrate a difference in the effect of PAA on the intranuclear localization of the 65K_{DBP} and ICP8 in that the 65K_{DBP} did not accumulate specifically to the prereplicative sites occupied by ICP8.

Similar double-labeling immunofluorescence experiments were performed to compare the localization pattern of Pol with that of the 65K_{DBP} and ICP8 in cells infected in the presence of PAA. Pol was detected with the rabbit antibody and the appropriate FITC-conjugated secondary antibody

FIG. 3. Effect of PAA on localization of 65K_{DBP}, Pol, and ICP8. Vero cells were infected with wt virus (20 PFU per cell) in the absence (A and B) or in the presence (C to H) of 400 μ g of PAA per ml and fixed at 7 h p.i. (A and B [same field]) Cells infected in the absence of PAA were stained with R219 (ICP8) IgG, FITC-conjugated anti-rabbit IgG, MAb 6898 (65K_{DBP}), and RITC-conjugated anti-mouse IgG. Cells were viewed with the green filter to detect ICP8 (A) or the red filter to detect the 65K_{DBP} (B). (C and D [same field]) Cells infected in the presence of PAA were stained and viewed as described for panels A and B to detect ICP8 (C) or the 65K_{DBP} (D). (E and F [same field]) Cells infected in the presence of PAA were stained with rabbit anti-Pol IgG, FITC-conjugated anti-rabbit IgG, MAb 39S (ICP8), and RITC-conjugated anti-mouse IgG. Cells were viewed with the green filter to detect Pol (E) and the red filter to detect ICP8 (F). (G and H [same field]) Cells infected in the presence of PAA were stained with anti-Pol IgG, FITC-conjugated anti-rabbit IgG, MAb 6898 (65K_{DBP}), and RITC-conjugated anti-mouse IgG. Slides were viewed with the green filter to detect Pol (G) and the red filter to detect the 65K_{DBP} (H).

and observed with the green filter (Fig. 3E and G), while ICP8 and 65K_{DBP} were detected with their respective mouse MAb and RITC-conjugated secondary antibodies and observed with the red filter (Fig. 3F and H, respectively). In the presence of PAA, Pol was found throughout the nucleus of infected cells but appeared more concentrated in some areas (Fig. 3E). Due to quenching of fluorescence during the 2-min exposure required for photography, only the most intense areas of localization are visible in the cells shown. Examination of the same cells for ICP8 localization revealed that these areas of concentration were the prereplication sites occupied by ICP8 (Fig. 3F). Nevertheless, the distribution of Pol in areas outside the prereplication sites was in contrast to the nearly exclusive localization of ICP8 to the prereplication sites in the nuclei of infected cells (see also Fig. 3C). When we examined infected cells grown in the presence of PAA and stained for both Pol and 65K_{DBP} (Fig. 3G and H, respectively), we confirmed that the pattern of Pol accumulation was distinct from that of the 65K_{DBP}, although the diffuse distribution of the 65K_{DBP} throughout infected cell nuclei (Fig. 3H) indicated that it was possible for at least some of the Pol and 65K_{DBP} to exist together. Thus, Pol appears to have some affinity for prereplication sites or elements associated with prereplication sites in the presence of PAA.

Effects of *ts* mutations in DNA replication genes on localization of 65K_{DBP}. It has been hypothesized that ICP8 is a major organizational protein which attracts other replication proteins to critical sites in preparation for viral DNA synthesis (5). Because it was possible that a chemical inhibitor such as PAA might block or interfere with the localization of Pol or the 65K_{DBP} through its direct interaction with Pol molecules, we examined the effect of mutations in different viral DNA replication genes on the localization pattern of the 65K_{DBP} compared with that of ICP8, whose distribution in cells infected by some *ts* mutants has been well documented (32, 33). We were particularly interested in determining whether or not the 65K_{DBP} localized to prereplicative sites in cells infected with DNA⁻ *ts* mutants.

Vero cells were infected with *ts* mutants and incubated continuously at either the pT (34°C) or the npT (39.7°C) for 6 to 8 h p.i. Because of the reduced level of antigen production at 39.7°C, the low strength of the rabbit polyclonal antibodies compared with the mouse MAb available, and the limited wavelengths of light available for viewing, double-labeling immunofluorescence experiments were not possible for most of the mutants tested. Therefore, cell culture chambers were stained independently for the production of either 65K_{DBP} with MAb 6898 (Fig. 4) or ICP8 with MAb 39S (Fig. 5) unless otherwise noted. No qualitative difference was observed in the pattern of antigen accumulation at 6 or 8 h p.i. in any of the mutant-infected cells.

In cells infected with a DNA⁺ control mutant, *ts*J12, containing a mutation in the gene for glycoprotein B, the 65K_{DBP} (which is wt in *ts*J12) localized to replication compartments at both the pT and npT (Fig. 4A and F, respectively). A similar localization pattern for the 65K_{DBP} was observed in wt virus-infected cells at 34, 37, and 39.7°C (Fig. 1) (10; data not shown). ICP8 was also found in replication compartments at both the pT and npT in cells infected with *ts*J12 or the wt virus (data not shown). However, in cells infected with each of the DNA⁻ mutants, the 65K_{DBP} (Fig. 4B to E) and ICP8 (Fig. 5A and C; data not shown) localized to replication compartments only at the pT.

However, at the npT, the localization patterns of the 65K_{DBP} and ICP8 were different in cells infected with some

of the mutants but the same or similar for others. For example, in cells infected with each of the ICP8 *ts* mutants and incubated at 39.7°C, the 65K_{DBP} (which is wt in *ts*A16- and *ts*A24-infected cells) concentrated in the nucleus (excluding the nucleoli) but did not localize to discrete areas within the nucleus. This staining pattern was therefore referred to as diffuse and resembled the 65K_{DBP} staining pattern in cells infected with wt virus and grown in the presence of PAA (compare Fig. 4H and I with Fig. 3D). The ICP8 MAb 39S was not capable of detecting the mutant form of ICP8 at the npT. However, staining with the rabbit polyclonal antibody revealed that ICP8 remained in the cytoplasm in cells infected with *ts*A24 at the npT and localized diffusely within the nucleus in *ts*A16-infected cells (data not shown). This diffuse localization pattern for ICP8 has been observed previously in cells infected with ICP8 mutants (32). Thus, these results are consistent with the hypothesis that functional ICP8 is required for localization of replication proteins to prereplicative sites under conditions nonpermissive for viral DNA synthesis. They further demonstrated, however, that functional ICP8 is not necessary for the localization of the 65K_{DBP} to the nucleus since the 65K_{DBP} accumulated in the nuclei of cells infected with either of the ICP8 mutants and incubated at the npT.

In cells infected with the 65K_{DBP} *ts* mutant *ts*701, the defective 65K_{DBP} was capable of migrating to the nucleus and exhibited a punctate pattern of intranuclear staining (Fig. 4G). However, these punctate structures were larger and not as evenly dispersed throughout the nucleus as the punctate prereplicative sites in which ICP8 was localized in PAA-treated wt-infected cells (Fig. 3C and F) or in the *ts*701-infected cells at the npT (Fig. 5B). However, ICP8 also accumulated in larger structures within some cells displaying dispersed prereplicative sites. Double-labeling experiments with the R219 ICP8 antibody and the 65K_{DBP} MAb 6898 indicated that the punctate sites in which the 65K_{DBP} localized in *ts*701 mutant-infected cells at the npT (Fig. 6B) were not the same as the prereplicative sites occupied by ICP8 (Fig. 6A). Due to the small size and general distribution of sites occupied by ICP8, we cannot exclude the possibility of some overlap with the sites occupied by the 65K_{DBP}, although the overall patterns of distribution of these two proteins were clearly distinct in these cells. Therefore, the presence of wt ICP8 at prereplicative sites is not sufficient to attract the mutant form of the 65K_{DBP}. However, on the basis of these results alone, we could not exclude the possibility that the failure of the 65K_{DBP} to localize to the prereplicative sites was due directly to defects in the conformation of the 65K_{DBP} in these cells.

Therefore, the pattern of 65K_{DBP} was determined in cells infected with the Pol mutant, *ts*D9, in which both the 65K_{DBP} and ICP8 were wt at the npT. The pattern of 65K_{DBP} localization in the Pol mutant was intermediate between that observed in ICP8 or 65K_{DBP} mutant-infected cells at the npT. Specifically, the 65K_{DBP} staining pattern in *ts*D9-infected cells was characterized by lightly stained punctate structures against a generally diffusely stained nuclear background (Fig. 4J). The pattern of ICP8 accumulation in *ts*D9-infected cells at the npT (Fig. 5D) was indistinguishable from that observed in PAA-treated wt virus-infected cells (Fig. 3D) in that most of the ICP8 concentrated in the prereplicative sites with little or no diffuse staining in the nucleus. Because of lack of visual detail in double-labeling immunofluorescence experiments in *ts*D9-infected cells maintained at the npT (data not shown), we cannot exclude the possibility that the punctate sites occupied by the

FIG. 5. Localization of ICP8 in *ts* mutant-infected cells. Vero cells were infected with *ts701* (A and B) or *tsD9* (C and D) at an MOI of 20 PFU per cell and incubated at 34°C (A and C) or 39.7°C (B and D). Cells were fixed at 8 h p.i., stained with MAb 39S and FITC-conjugated goat anti-mouse IgG, and viewed with the green filter set.

65K_{DBP} represent a subset of those occupied by ICP8. However, it is equally possible that the punctate sites represent areas of accumulation which are distinct from those of ICP8, such as was shown for the *ts701* mutant (Fig. 6). Taken together, these results indicate that functional ICP8 is not sufficient to organize the 65K_{DBP} to prereplicative sites in the absence of viral DNA synthesis and suggest that other factors (e.g., Pol or Pol-dependent activity) are involved in localizing the 65K_{DBP} in preparation for viral DNA replication.

Continuous viral DNA synthesis is required to maintain 65K_{DBP} in replication compartments. As indicated by the experiments described above, the 65K_{DBP} does not accumulate in prereplication or replication compartments when infected cells are maintained continuously under conditions which prevent viral DNA synthesis. We next attempted to determine whether the 65K_{DBP}, once localized in replication compartments, would continue to be detectable in these compartments when viral DNA replication ceased. We therefore incubated cells infected with either the 65K_{DBP} mutant, *ts701*, or the Pol mutant, *tsD9*, at the pT until 8 h p.i. At that time, cultures were shifted up to the npT and cells were fixed and stained at intervals thereafter with the 65K_{DBP} MAb 6898 (Fig. 7).

At 8 h p.i. (i.e., before temperature shift), substantial quantities of the 65K_{DBP} had accumulated in replication compartments in the nuclei of cells infected with either *ts701* or *tsD9* (Fig. 7A and E). As early as 1 h after temperature shift-up, 65K_{DBP}-specific staining was distinct from that observed before shift-up in that staining was more punctate and less intense (Fig. 7B and F). Similarly, the 65K_{DBP} was more diffuse in infected cells 2 h postshift, although some small accumulations reminiscent of replication compartments were occasionally observed (Fig. 7C and G). However, by 3 h postshift, the patterns of 65K_{DBP} localization in both *ts701* and *tsD9* mutant-infected cells were indistinguishable from those observed in cells maintained continuously at 39.7°C (compare Fig. 7D and H with Fig. 4G and J).

These results indicated that continuous viral DNA synthesis was required to maintain the 65K_{DBP} within replication compartments. However, a remaining possibility was that the apparent change in localization of the 65K_{DBP} in *ts701*-infected cells was due to aberrant localization of newly synthesized mutant protein after temperature shift-up. When cells were shifted in the presence of cycloheximide (50 µg/ml), the pattern of 65K_{DBP} localization (Fig. 8) was similar to that observed in cells maintained continuously at the npT (Fig. 4G) and in cells shifted up to the npT in the

FIG. 4. Localization of the 65K_{DBP} in *ts* mutant-infected cells. Vero cells were infected with each mutant (20 PFU per cell) and incubated at the pT of 34°C (A through E) or the npT of 39.7°C (F through J). Cells were fixed at 6 or 8 h p.i. and stained with MAb 6898 and FITC-conjugated secondary antibody to detect the 65K_{DBP}. All fields were photographed with the green filter set. (A and F) *tsJ12*; (B and G) *ts701*; (C and H) *tsA16*; (D and I) *tsA24*; (E and J) *tsD9*.

FIG. 6. 65K_{DBP} and ICP8 do not colocalize in *ts701*-infected cells at the npT. Vero cells were infected with *ts701* (MOI of 20 PFU per cell), incubated at 39.7°C, and harvested at 5 h p.i. Cells were fixed and stained with the R219 (ICP8) antibody, FITC-conjugated anti-rabbit IgG, anti-65K_{DBP} MAb, and RITC-conjugated anti-mouse IgG. The figure shows the same cell viewed with the green filter to detect ICP8 (A) or the red filter to detect the 65K_{DBP} (B).

absence of cycloheximide (Fig. 7D). Thus, the change in localization of the 65K_{DBP} after temperature shift-up was due largely to the relocation of existing protein.

DISCUSSION

The proteins ICP8, Pol, and 65K_{DBP} are required for the replication of viral DNA during productive infection by HSV-1 (11, 19, 35, 39) and for origin-dependent DNA replication of plasmids in transfection assays (40). Numerous biochemical and immunological studies have indicated that many of the proteins involved in viral DNA replication are associated with complexes (8, 14, 15, 28, 29, 38). Interestingly, single-label immunofluorescence studies have revealed that during productive infection, four of the seven replication proteins described by Wu and co-workers (40) appear to localize to structures which resemble replication compartments (25), suggesting that at least these four proteins (ICP8, Pol, 65K_{DBP}, and UL9) exist as a complex.

In this report, we extended previous immunofluorescence data to demonstrate that the 65K_{DBP} colocalizes with ICP8 (Fig. 1) and Pol (Fig. 2) to replication compartments during productive infection. By contrast, under conditions nonpermissive for viral DNA replication (i.e., in the presence of PAA [Fig. 3] or in *ts* mutant-infected cells grown at the npT [Fig. 4 to 6]), neither the 65K_{DBP} nor ICP8 localized to replication compartments. However, in each case, the 65K_{DBP} localized exclusively to the nuclei of infected cells. These results are consistent with transfection studies which demonstrate that the 65K_{DBP} expressed from plasmids localizes diffusely within nuclei, sparing nucleoli (unpublished data). These studies further indicate that the nuclear localization of the 65K_{DBP} is an intrinsic property of the protein and does not require other virus-encoded functions for its transport to the nucleus.

We also found that the 65K_{DBP}, once localized to replication compartments, redistributed after temperature shift-up of *ts701*- and *tsD9*-infected cells from the pT to the npT (Fig. 7). The redistribution process was not due to degradation of existing protein followed by aberrant localization of protein synthesized *de novo* since it occurred extremely rapidly (within 1 h after temperature shift; Fig. 7B and F) and because the same redistribution was observed after temperature shift-up in the presence of cycloheximide (Fig. 8). Not all viral proteins known to localize to replication compart-

ments undergo redistribution once viral DNA synthesis is blocked, inasmuch as ICP4, a major HSV transcriptional regulatory protein, remains in replication compartments after inhibition of viral DNA synthesis (12).

We were surprised to find that the 65K_{DBP} did not always colocalize with ICP8 in the absence of viral DNA synthesis. It has been suggested that ICP8 functions as the major organizational protein which directs viral and cellular replication proteins to prereplicative sites which are most likely associated with the nuclear matrix (5). Consistent with the findings of these investigators, we found that when ICP8 itself was defective but retained the ability to enter the nucleus, as in the ICP8 mutant *tsA16* grown at the npT, the ICP8 failed to accumulate in prereplicative sites but was diffusely localized throughout the nuclei of infected cells (data not shown). The 65K_{DBP} in both of the ICP8 mutant-infected cells grown at the npT exhibited a similar diffuse nuclear localization pattern (Fig. 4H and I). Additionally, in support of the hypothesis, we found Pol to accumulate in prereplicative sites occupied by ICP8 in cells infected in the presence of PAA (Fig. 3E and F).

However, several pieces of data presented in this report fail to support the hypothesis, at least with respect to the 65K_{DBP}. In wt virus-infected cells grown in the presence of PAA, ICP8 was found predominantly to be associated with punctate prereplicative sites (Fig. 3B), although the 65K_{DBP} was diffusely localized in the nuclei, failing to concentrate to the prereplicative sites occupied by ICP8 (Fig. 3C). Moreover, in *ts701* (65K_{DBP}) mutant-infected cells grown at the npT, we demonstrated that the 65K_{DBP}, detected in larger punctate structures, again failed to colocalize with ICP8 to prereplicative sites (Fig. 6). Taken together, our results demonstrate that the presence of functional ICP8 is not sufficient to organize the 65K_{DBP} to prereplicative sites. However, ICP8 may act in concert with other proteins to direct the replication proteins to sites in preparation for DNA synthesis. Indeed, we found that Pol was directed to these sites, although not exclusively so, in the presence of PAA (Fig. 3).

Several studies indicate that the 65K_{DBP} and Pol are closely associated (8, 38) and that the 65K_{DBP} is a Pol accessory protein (7). Therefore, we expected that these two proteins would colocalize in infected cells even under conditions nonpermissive for virus replication. However, we

FIG. 7. Effect of temperature shift-up on the localization of 65K_{DBP} in *ts701* and *tsD9* mutant-infected cells. Vero cells were infected with *ts701* (A through D) or *tsD9* (E through H) at an MOI of 20 PFU per cell and incubated at 34°C until 8 h p.i., at which time the cultures were shifted to 39.7°C. At the time of shift (0 h), a set of cultures was fixed (A and E), and additional sets of cultures were fixed at 1 h (B and F), 2 h (C and G), or 3 h (D and H) after temperature shift-up. Fixed cells were stained with MAb 6898 and FITC-conjugated goat anti-mouse IgG to detect the 65K_{DBP}.

FIG. 8. Relocalization of the 65K_{DBP} after temperature shift-up in the absence of de novo protein synthesis. Vero cells were infected with *ts701*, maintained at 34°C until 8 h p.i., and then shifted to 39.7°C. Cycloheximide (50 µg/ml) was added at the time of temperature shift-up to inhibit protein synthesis, and cells were harvested at various times thereafter and stained as described in the legend to Fig. 7 to detect the 65K_{DBP}. A sample field of cells fixed at 2 h postshift is shown.

observed a difference in the localization pattern of the 65K_{DBP} and Pol in the presence of PAA. Although much of the Pol concentrated in prereplication sites (Fig. 3E), the 65K_{DBP} was diffusely localized in the nuclei of the same infected cells (Fig. 3F). In fact, we have tested a variety of 65K_{DBP} MAb concentrations and find no evidence of specific areas of intranuclear accumulation of 65K_{DBP} under these conditions even in the presence of limiting antibody (data not shown). Nevertheless, the additional presence of Pol in areas outside prereplication sites indicated the possibility that at least some of the Pol and 65K_{DBP} was associated in the presence of PAA. It is also possible that the binding of PAA to Pol interfered with the ability of the 65K_{DBP} to interact with Pol molecules, thus causing the diffuse localization pattern. However, the additional failure of the 65K_{DBP} to localize to prereplication sites in the *ts701* and *tsD9* mutant-infected cells maintained at the npT argues against a PAA-specific artifact. Because of the low abundance of Pol in DNA⁻ *ts* mutant-infected cells, it has not been possible with these cells to perform colocalization studies of Pol with other replication proteins using the Pol antibody available to us. Thus, identification of the conditions under which Pol and the 65K_{DBP} associate in *ts* mutant-infected cells at the npT must await the development of a more potent Pol antibody or a more sensitive method for detecting the complexes.

Active replication fork progression may also be necessary for ICP8, Pol, 65K_{DBP}, and perhaps other replication proteins to form stable associations. Precedence for multiple replication protein associations which are unstable exists in *E. coli* in which the beta subunit of the DNA polymerase III holoenzyme cannot be isolated as part of the multiprotein complex in the absence of ATP (13, 23). It is thought that the subunit associates with the core polymerase subunit by virtue of its interactions with other polymerase accessory proteins (22). It will be necessary to transfect cells containing an HSV-1 origin of replication sequentially with the various replication genes to determine both the order in which the proteins associate and the requirement for replication fork progression. It also will be interesting to determine whether alterations in some of the other proteins required for origin-dependent DNA replication simulta-

neously affect the localization of the 65K_{DBP}, Pol, and other proteins required for the replication of HSV DNA.

ACKNOWLEDGMENTS

We thank Ann Cross, Howard Marsden, Martin Zweig, Paul Olivo, and Mark Challberg for the generous gifts of antibodies used in these studies and George Milo for the use of his fluorescence microscope. We also thank David Knipe for discussions of his Pol localization studies prior to publication and Merle Potchinsky for technical assistance in the characterization of the Pol antibody.

This work was supported in part by Public Health Service grants GM 34930 and the O.S.U. Comprehensive Cancer Center core grant CA 16958 (D.S.P.), AI 28537 (P.A.S.), and AI 07282 (D.I.D.) from the National Institutes of Health, by grant MV-317 from the American Cancer Society (D.S.P.), by a fellowship from the Medical Foundation in Boston (D.I.D.), and by a Baxter Life Sciences Foundation award (C.S.C.).

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