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Association of Herpes Simplex Virus Regulatory Protein ICP22 with Transcriptional Complexes Containing EAP, ICP4, RNA Polymerase II, and Viral DNA Requires Posttranslational Modification by the U_L13 Protein Kinase

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The expression of herpes simplex virus 1 γ (late) genes requires functional α proteins (γ_1 genes) and the onset of viral DNA synthesis (γ_2 genes). We report that late in infection after the onset of viral DNA synthesis, cell nuclei exhibit defined structures which contain two viral regulatory proteins (infected cell proteins 4 and 22) required for γ gene expression, RNA polymerase II, a host nucleolar protein (EAP or L22) known to be associated with ribosomes and to bind small RNAs, including the Epstein-Barr virus small nuclear RNAs, and newly synthesized progeny DNA. The formation of these complexes required the onset of viral DNA synthesis. The association of infected cell protein 22, a highly posttranslationally processed protein, with these structures did not occur in cells infected with a viral mutant deleted in the genes U_L13 and U_S3, each of which specifies a protein kinase known to phosphorylate the protein.

The genes encoded by the herpes simplex virus 1 (HSV-1) genome have been classified into major groups, designated α, β, γ_1, and γ_2, whose expression is coordinately regulated and sequentially ordered in a cascade fashion (14, 15). The α genes encode largely regulatory proteins and are expressed in the absence of prior viral protein synthesis. The products of the α genes enable expression of the β genes by mechanisms as yet unknown. The expression of γ genes, which encode mainly structural viral proteins, requires the expression of α and β genes and is partially (γ_1 genes) or totally (γ_2 genes) dependent on the onset of viral DNA synthesis (reviewed in reference 40). The mechanism by which expression shifts from α and β to γ_1 and especially to γ_2 genes is not well understood. We report that late in infection, the products of two α genes, infected cell proteins 4 and 22 (ICP4 and ICP22), are present in discrete nuclear structures which contain a cellular protein designated EBER-associated protein (EAP), the cellular RNA polymerase II (RNA pol II), and newly synthesized viral DNA. Colocalization of these molecules in a nuclear structure dependent for its formation on the onset of viral DNA synthesis suggests that these complexes are functional centers for transcription of γ genes. Relevant to this report are the following.

(i) ICP4 is present in two copies per genome (Fig. 1) and acts both as a transactivator and as a repressor (8, 31, 40). The response elements for the repressor functions of this protein are high-affinity binding sites located near transcription initiation sites of the genes repressed by the protein (12, 19, 20, 26, 27, 29). The strength of repression is dependent on both the distance from and stereoaxial alignment with the TATA box (9, 21, 24), possibly due to interaction with TATA-binding protein and TFIIB (43). The response elements presumed to function in the transactivation of viral genes by ICP4, however, are not known. ICP4 binds to low-affinity sites on viral DNA, but no consensus or function for these sites has been reported (19, 20, 26, 28). Mutations in ICP4 may affect repression and activation independently of each other (42).

(ii) ICP22 (Fig. 1) appears to be dispensable for HSV-1 growth in some cell lines but not in others (30, 41). In most cell lines, deletions in this gene affect the expression of α0, a gene encoding a promiscuous transactivator, and of both the RNA and protein products of a subset of γ genes (32). Viral DNA is transcribed by RNA pol II (5, 40). The involvement of ICP22 in transcription of viral genes may also be inferred from the report that ICP22 is involved in the aberrant phosphorylation of RNA pol II to an intermediate state in some but not all cells (36, 37). ICP22 is highly posttranslationally processed. Modifications include guanylylation, adenylylation, and phosphorylation by the protein kinases U_L13 and U_S3 encoded by the virus (2, 32, 33).

The domain of the gene encoding ICP22 (α22) yields two polypeptides transcribed independently, i.e., the full-length ICP22 and its truncated version $U_S1.5$, whose amino acid sequence is identical to that of the carboxyl-terminal portion of ICP22 (3). Preliminary studies indicate that these two proteins colocalize (4).

(iii) EAP derived its name from its association with EBERs, small nonpolyadenylated RNAs of unknown function produced in Epstein-Barr virus-infected cells (44, 45). EAP was later found to be identical to L22, a component of the ribosomes (46). EAP resides in both nucleoli and ribosomes of uninfected cells. In Epstein Barr virus-infected B lymphocytes, a substantial portion of the nucleolus-associated EAP relocates into the nucleoplasm and colocalizes with EBERs (46). The first evidence that EAP binds to ICP4 emerged from an analysis of cellular proteins interacting with ICP4 in a yeast two-hybrid system (23). That this association appears to be significant emerged from the observations that in vitro, EAP disrupts the interaction of ICP4 with its cognate DNA sequences in a dose-dependent manner and that EAP colocalizes with ICP4 in discrete nuclear structures. The formation of these structures is precluded by phosphonoacetic acid, a specific inhibitor of viral DNA synthesis, and by incubation at a nonpermissive temperature of cells infected with an HSV-1

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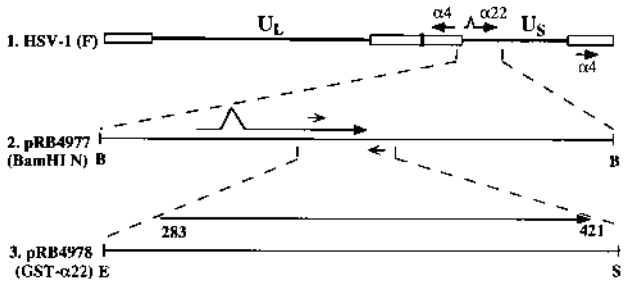

FIG. 1. Diagrammatic representation of the HSV-1 genome, the locations of the α4 and α22 genes, and a schematic representation of the domain of the α22 gene encoding the carboxyl-terminal region of ICP22 fused with GST and cloned into expression vector pGEX. Line 1, the thick lines represent the unique sequences U_L and U_S. The rectangles represent the repeat sequences flanking U_L and U_S. The locations and directions of transcription of the α4 and α22 genes are shown. Line 2, pRB4977 carries the *Bam*HI N fragment of HSV-1(F), which contains the entire α22 gene, and was the template DNA used for amplification of the desired sequence by PCR to clone the C-terminal 138 codons into the pGEX vector. Arrows represent positions of the sequences of oligonucleotides used for the PCR. Line 3, pRB4978 was obtained by cloning the PCR product into a pGEX vector to obtain a GST-ICP22$_{(283\text{-}431)}$ fusion protein. B, *Bam*HI; E, *Eco*RI; S, *Sal*I.

mutant (*ts*HA1) carrying a temperature-sensitive lesion in the ICP8 gene, necessary for viral DNA synthesis (18, 23).

(iv) Viral DNA is made by a rolling-circle mechanism (reviewed in reference 40). Progeny DNA consists of concatemers of numerous unit-length genomes arranged head-to-tail. Electron microscopy shows the concatemers to appear as dense tangles of viral DNA (16, 17). The proteins involved in viral DNA synthesis occupy a central compartment within the nucleus (6, 7, 38), distinct from the compartment containing proteins involved in assembly of mature and immature capsids (47).

The association of ICP4, ICP22, RNA pol II, and newly synthesized progeny viral DNA in spatially defined nuclear structures and the evidence that the formation of these structures is dependent on initiation of viral DNA synthesis suggest that these structures play a significant role in the expression of late genes whose transcription depends on the onset of viral DNA synthesis.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were obtained from the American Type Culture Collection. Vero cells were originally obtained from J. McClaren. The human foreskin fibroblasts (HFF) were originally obtained from George Kemble (Aviron, Mountainview, Calif.). HSV-1(F) is the prototype HSV-1 strain used in this laboratory (10). The recombinant virus R7353 contains deletions in both U_L13 and U_S3 genes (32). In recombinant HSV-1 R325, approximately 800 bp comprising the carboxyl-terminal half of the α22 gene were deleted (30). *d*120, derived from HSV-1(KOS), contains a deletion in both copies of the α4 gene and grows only in a Vero cell line expressing the α4 gene (4). Both the virus and the cell line were kind gifts of Neal DeLuca (University of Pittsburgh).

GST pulldown assays. HeLa cells were mock infected or infected with HSV-1(F), *d*120, or R325 at a multiplicity of infection of 10 PFU per cell. At times indicated for individual experiments, the cells were harvested, pelleted by low-speed centrifugation, rinsed once in phosphate-buffered saline (PBS), and resuspended at a concentration of 10^7 cells per ml of a lysis solution consisting of PBS containing 1% Nonidet P-40, 1% deoxycholate, and the protease inhibitors tolylsulfonylphenylanalyl chloromethyl ketone (TPCK; 10 μM) and α-tosyl-L-lysine chloromethyl ketone (TLCK; 10 μM). The glutathione *S*-transferase (GST) or GST-EAP fusion proteins, originally obtained from Joan Steitz (Yale University), were expressed in *Escherichia coli* BL21 cultures and prepared from bacterial pellets according to published procedures (23). For the pulldown experiments, cell extracts from approximately 2×10^7 cells in 200 μl of lysis buffer were mixed with 100 μl of slurry containing 50% glutathione-agarose beads to which the GST or GST-EAP proteins were bound. The mixture was allowed to react for 3 to 4 h at 4°C. The beads were then rinsed in PBS three times, and the bound proteins were solubilized in a buffer containing sodium dodecyl sulfate, subjected to electrophoresis in a denaturing gel, transferred electrically to a nitrocellulose sheet, and reacted with antibody.

Antibodies. Monoclonal antibodies H640 to ICP4 and H1113 to ICP27 were obtained from the Goodwin Institute for Cancer Research Inc. (Plantation, Fla.). Monoclonal antibodies to the C-terminal domain of the larger subunit of RNA pol II and to bromodeoxyuridine (BUdR) were obtained from Promega and Sigma Chemical Co. (St. Louis, Mo.), respectively. A rabbit polyclonal antiserum (R77) that reacts with an amino acid sequence located at the amino terminus of ICP22 has been previously described (1). An additional rabbit polyclonal antiserum to a bacterial fusion protein consisting of GST fused to the carboxyl-terminal 138 amino acids of ICP22 was generated as a part of this study (see Results). Immunofluorescence assays of ICP22 were done with a mixture of the two rabbit polyclonal antisera. The goat anti-rabbit and anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibodies were purchased from Sigma. The goat anti-rabbit and anti-mouse Texas red-conjugated antibodies were purchased from Molecular Probes, Inc. (Eugene, Oreg.).

Immunofluorescence. Approximately 5×10^4 HEp-2 cells or HFF were seeded on wells on glass slides (Cell-line Inc., Newfield, N.J.) and incubated overnight at 37°C. The cells were then exposed to 10 PFU of wild-type or mutant virus per cell. At 4 or 8 h after infection, the cells were fixed in ice-cold methanol, air dried, and reacted with PBS containing 1% bovine serum albumin and 20% normal human serum for 1 h at room temperature to saturate viral Fc receptors responsible for nonspecific binding of immunoglobulin G (IgG) to infected cells. The cells were then reacted for 2 h at room temperature with primary antibody diluted in PBS containing 1% bovine serum albumin and 10% human serum, rinsed extensively with PBS, and reacted for 1 h with a secondary antibody conjugated to either FITC or Texas red. The slides were again rinsed extensively and mounted in 95% glycerol in PBS containing 1 mg of *p*-phenylenediamine per ml to reduce fading of the FITC signal. The slides were examined under a Zeiss confocal fluorescence microscope; digitized images of the fluorescent antibody-stained cells were acquired with software provided with the confocal microscope and printed by a Tektronix Phaser 440 printer. Single-color images were acquired by excitation, using an argon/krypton laser at 488 nm (FITC) or 568 nm (Texas red). Double-stained images were obtained by acquiring a split image of both fluorochromes filtered by 515- to 540-nm band pass (FITC) and 590-nm long-pass (Texas red) filters and subsequent overlay of the two color images. Identical patterns were observed for single fluorochromes, indicating that the filters used eliminated any leakage of fluorescent signals between red and green channels.

In vivo labeling of DNA with BUdR. Infected cells grown on microscope slides were incubated at 8 h after infection for 30 min at 37°C with 10 μM BUdR in Dulbecco modified Eagle medium containing 5% newborn calf serum. Cells were rinsed with PBS, fixed in ice-cold methanol, and then treated for 10 min with 4 N HCl to expose the incorporated BUdR residues. The cells were washed two times for 10 min each in PBS prior to staining with anti-BUdR antibody.

RESULTS

Preparation and characterization of a rabbit antiserum to the carboxyl-terminal portion of ICP22. A rabbit polyclonal antiserum to ICP22 was generated by immunization of rabbits with a chimeric protein consisting of the carboxyl-terminal 138 amino acids of ICP22 fused to GST (Fig. 1). The antibodies produced following inoculation of rabbits with the GST-ICP22 fusion protein reacted with a series of proteins with apparent M_rs ranging from approximately 67,000 to 72,000. These bands represent the five isoforms of ICP22 (Fig. 2, lanes 3 and 4). The antibody did not react with any protein present in mock-infected cell lysates of rabbit skin cells or Vero cells (Fig. 2, lanes 1 and 2). The preimmune sera did not react with any protein present in either uninfected or infected cell lysates (data not shown).

EAP interacts with ICP22 in vitro. The purpose of the next series of experiments was to determine whether HSV-1 immediate-early proteins other than ICP4 interact in vitro with EAP. Infected cell extracts were mixed with either GST-EAP fusion protein or GST alone conjugated to agarose beads, and protein complexes bound to the beads were subjected to electrophoresis on denaturing gels, transferred to a nitrocellulose membrane, and reacted with antibodies to three HSV-1α proteins, ICP4, ICP22, and ICP27. As expected, ICP4 was detected in the extracts pulled down by GST-EAP but not by GST alone. ICP22 was also detected in the same membrane, pulled down by GST-EAP but not by GST alone (Fig. 3). ICP27, however, was not detected (data not shown).

One hypothesis that could account for these results is that

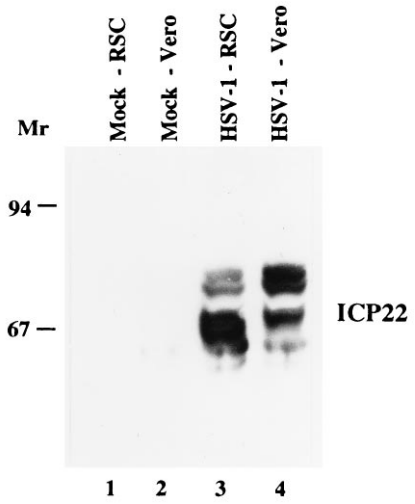

FIG. 2. Photograph of an immunoblot showing the reactivity of a rabbit antiserum generated to ICP22. Cell lysates from either mock-infected (lanes 1 and 2) or HSV-1(F)-infected (lanes 3 and 4) rabbit skin cells (RSC; lanes 1 and 3) or Vero cells (lanes 2 and 4) were electrophoretically separated on a denaturing 10% acrylamide gel, electrophoretically transferred to a nitrocellulose membrane, and reacted with a 1:500 dilution of the rabbit antiserum and subsequently with anti-rabbit IgG conjugated to alkaline phosphatase and the phosphatase substrate. M_rs are in thousands.

ICP22 was brought down by the ICP4 bound to GST-EAP. To test this hypothesis, the experiment was repeated with lysates of cells infected with wild-type virus or with the recombinant *d*120, which lacks both copies of the α4 gene (8). These experiments showed that both ICP4 and ICP22 proteins interact specifically and independently with EAP (Fig. 3).

The purpose of the next series of experiments was to map the approximate domain of ICP22 which bound the GST-EAP fusion protein. The experiment was therefore repeated with lysates of cells infected with the recombinant virus R325, which

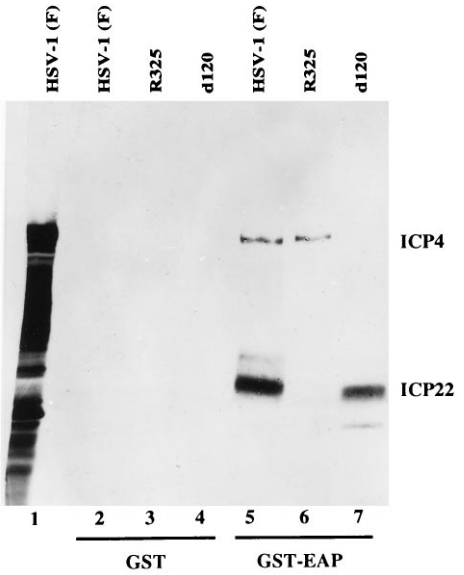

FIG. 3. Photograph of proteins from lysates of cells infected with HSV-1(F), R325, or *d*120 captured by GST or GST-EAP bound to beads, solubilized in a solution containing sodium dodecyl sulfate, electrophoretically separated in a denaturing 8.3% polyacrylamide gel, electrically transferred to a nitrocellulose membrane, and reacted with antibodies to ICP4 and to ICP22.

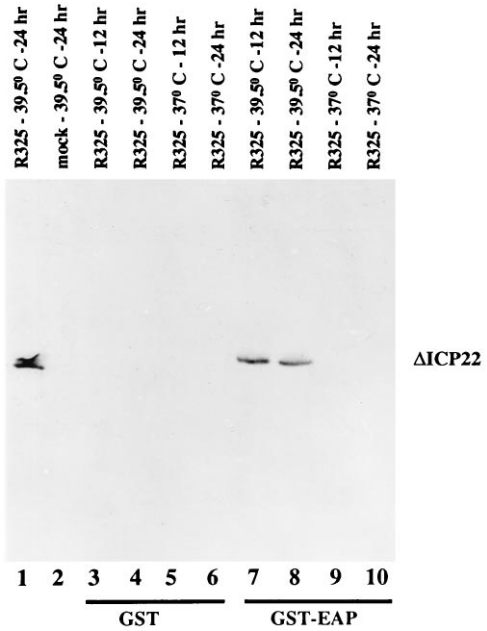

FIG. 4. Photograph of truncated ICP22 captured by GST or GST-EAP bound to beads from lysates of cells mock infected or infected with R325 and incubated at either 37 or 39.5°C. The proteins captured by the beads were solubilized, electrophoretically separated in a denaturing 12% polyacrylamide gel, electrically transferred to a nitrocellulose membrane, and reacted with rabbit polyclonal antibody R77 directed to a peptide located in the amino-terminal portion of ICP22.

encodes the amino-terminal 200 amino acids but lacks the carboxyl half of the coding domain of ICP22. The truncated form of ICP22 is not phosphorylated by the viral kinases U_L13 and U_S3 and is readily degraded at 37°C. The protein is stable in infected cells maintained at 39.5°C. In pulldown experiments as described above, GST-EAP specifically brought down the truncated ICP22 (Fig. 4, lanes 7 and 8). In addition to the demonstration that EAP interacts with the amino-terminal 200 amino acids of ICP22, this result also shows that viral phosphorylation of ICP22 is not required for the interaction between EAP and ICP22.

ICP4 and ICP22 colocalize in the nuclei of infected cells. ICP4 is diffusely distributed throughout the infected cell nucleus early in infection but forms discrete dense bodies after the onset of viral DNA replication (18, 23). This laboratory reported earlier that EAP, which localizes to nucleoli in uninfected cells and early in HSV-infected cells, is redistributed late in infection to the dense bodies defined by the presence of ICP4 (23). The results of the pulldown experiments detailed above suggested that ICP22 may also localize to these dense bodies. Because our earlier studies were done in HEp-2 cells, we examined the distribution of ICP4 and ICP22 proteins in HEp-2 cells and found that the two proteins colocalized in the dense bodies (Fig. 5b). Colocalization of ICP4 and EAP in HEp-2 cells is also shown (Fig. 5a).

ICP22 and RNA pol II colocalize in the nuclei of infected cells. The objective of the experiments described in this section was to determine whether the nuclear structures described above contain cellular factors required for transcription of viral genes. In the first series of experiments, GST-EAP pulldown experiments similar to those described above were done to determine whether EAP binds RNA pol II. In these experiments, we failed to detect RNA pol II in immunoblots of proteins bound by GST-EAP (data not shown).

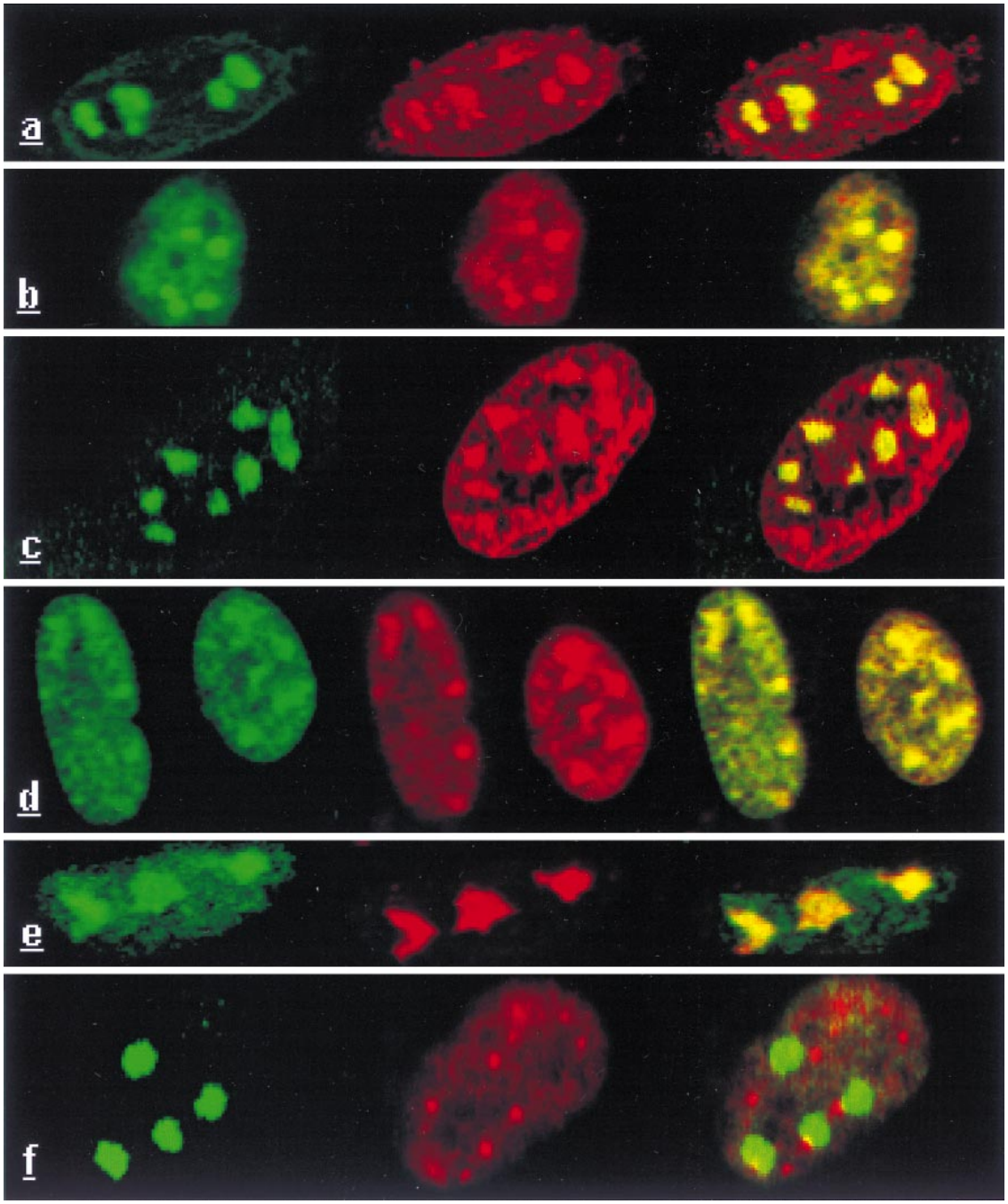

FIG. 5. Confocal, digital images of representative infected cells reacted with antibodies to viral or cellular proteins. The infected cells were fixed and stained 8 h after infection, reacted with a combination of monoclonal and polyclonal antibodies, rinsed, and then reacted with anti-mouse IgG or anti-rabbit IgG conjugated to FITC (green fluorescence) or to Texas red (red fluorescence). The antibodies were monoclonal antibodies to ICP4, RNA pol II, or BUdR and polyclonal rabbit sera to IC22 or EAP. Single-color images were captured separately and are shown in the left and middle columns; the two colors were then overlaid and are shown in the right column. The yellow color visualized in the overlaid images represents colocalization of red and green fluorescence. The images were captured with software provided by Zeiss with the confocal microscope, stored on an optical disk, and subsequently printed by a Tektronix Phaser 440 color printer, using Adobe Photoshop software. (a) HEp-2 cells infected with HSV-1(F) and reacted with anti-ICP4 (FITC) and anti-EAP (Texas red); (b) HEp-2 cells infected with HSV-1(F) and reacted with anti-ICP4 (FITC) and anti-ICP22 (Texas red); (c) HFF infected with HSV-1(F) and reacted with anti-ICP4 (FITC) and anti-ICP22 (Texas red); (d) HFF infected with HSV-1(F) and reacted with anti-ICP22 (FITC) and anti-RNA pol II (Texas red); (e) HFF infected with HSV-1(F) and reacted with anti-ICP22 (FITC) and anti-BUdR (Texas red); (f) HFF infected with R7353 (U_L13^-/U_S3^-) and reacted with anti-ICP4 (FITC) and anti-ICP22 (Texas red).

In the second series of experiments, we reacted infected HFF with rabbit polyclonal antibodies to ICP22 and monoclonal mouse antibodies to RNA pol II. The distribution of RNA pol II and of ICP22 was examined with the aid of confocal microscopy. The choice of the cells used in this study was dictated by the observation reported elsewhere that ICP22 plays a key role in viral replication in these cells (30, 41). The formation of ICP4 dense bodies and the colocalization of ICP4 and ICP22 in HFF was comparable to that observed in HEp-2 and in all other cell lines tested (Fig. 5c and unpublished

observations). As shown in Fig. 5d, the majority of RNA pol II (red fluorescence) colocalized with ICP22 (green fluorescence) in the dense nuclear bodies formed after the onset of viral DNA replication, while a minor fraction of both proteins remained diffusely distributed throughout the nucleus. Taken together, our results show that the dense nuclear bodies defined by the presence of ICP4 also contain ICP22, RNA pol II, and EAP.

The late HSV-1 transcriptional complexes defined by the presence of ICP4, ICP22, and EAP also contain progeny viral DNA. The observations that the dense nuclear bodies form only after the onset of DNA replication and contain transcriptional factors suggested the possibility that progeny viral DNA was present in these bodies. To test this hypothesis, HFF were incubated in medium containing BUdR for 30 min at 8 h after infection with HSV-1(F). The infected cells were then fixed and stained with an antibody to BUdR and analyzed by confocal immunofluorescence microscopy. As shown in Fig. 5e, the pattern of staining obtained with an antibody to BUdR (red fluorescence) completely overlapped the majority of ICP22 (green fluorescence) localized in the dense bodies.

Posttranscriptional modification by viral protein kinases is required for the localization of ICP22 to transcriptional compartments in HFF. The purpose of this series of experiments was to determine whether the posttranscriptional processing of ICP22 mediated by the viral protein kinases U_L13 and U_S3 is required for colocalization with ICP4. Cultures of HFF were prepared as described above and infected with either wild-type virus (Fig. 5c) or recombinant R7353, which lacks both U_L13 and U_S3 genes (Fig. 5f). In contrast to colocalization of ICP22 and ICP4 in dense bodies of nuclei of cells infected with wild-type virus, in cells infected with the recombinant virus R7353, ICP22 was largely dispersed in the nucleoplasm and formed dense structures smaller than and not overlapping with the nuclear dense bodies containing ICP4 (Fig. 5f). Some of these small structures formed by ICP22 were adjacent to the larger bodies formed by ICP4.

We also repeated the same experiments but using a U_L13^- virus and a recombinant in which the U_L13 sequence was repaired. We found that in cells infected with the U_L13^- virus, ICP22 failed to localize with ICP4 late in infection, whereas in cells infected with the repaired virus, the nuclear localization of ICP22 and ICP4 was indistinguishable from that observed with the wild-type HSV-1(F) parent (data not shown). We conclude from these results that U_L13 is required for the localization of ICP22 to the nuclear dense bodies.

DISCUSSION

In this report we show that ICP4, ICP22, EAP, and RNA pol II colocalize with progeny viral DNA in defined nuclear structures, that ICP22 interacts independently with EAP, and that this colocalization is dependent on the presence of functional viral protein kinases. As detailed in the introduction and in part below, both ICP4 and ICP22 have been shown to be involved in transcription of viral DNA. This is the first demonstration of both physical and functional presence of ICP22 in a nuclear compartment containing transcriptional factors at the time of transcription of γ genes. The salient features and relevant points arising from this report are as follows.

(i) Several laboratories have shown that the nuclear compartments that we have identified contain newly made viral DNA and factors necessary for transcription of viral genes. The two novel components of these structures that we have identified are EAP and ICP22. EAP presents a puzzle. Earlier, this laboratory reported that EAP interacts with ICP4 and that the chimeric protein GST-EAP but not GST alone disrupts the interaction of ICP4 with its cognate site on the DNA. In this study, EAP has been shown to interact with ICP22 independently of ICP4 in experiments involving the pulldown of ICP22 from cells infected with a virus lacking the α4 gene. None of the previous reports on EAP, however, suggest a role for this protein in the transcription of cellular or viral genes. The hypotheses most attractive at the moment are that (a) EAP provides the bond and enables a functional interaction of ICP4 and ICP22 with each other or with cellular factors and (b) EAP enables the transport of viral mRNA from transcriptional sites. While the hypothesis that EAP disrupts the binding of ICP4 to its cognate site and therefore derepresses late gene expression cannot be ruled out, we should note that the genes known to be repressed by ICP4, i.e., α4 and the open reading frame P, are not derepressed late in infection (14, 22, 27, 31).

(ii) Several studies predicted a role for ICP22 in transcription of viral genes. ICP22 appears to enhance the expression of at least one α (α0) and a subset of γ genes observed at both the mRNA and protein levels (32). ICP22 is extensively posttranslationally modified. The proteins that contribute to these modifications and produce readily identifiable forms of ICP22 are the viral protein kinases encoded by U_L13 and U_S3 (2, 32). The phenotype of the mutants lacking an intact ICP22 suggested that ICP22 may express functions both early and late in infection (32, 41). This conclusion is consistent with the observation that both U_L13 and U_S3 proteins are made after the synthesis of ICP22 and that modification of ICP22 by one or both kinases is required for colocalization with viral DNA, ICP4, EAP, and RNA pol II. Lastly, ICP22 has been reported to be involved in the posttranslational modifications of RNA pol II, and this finding is consistent with our results showing that these proteins colocalize at some point during the infectious cycle. As noted above, ICP22 is dispensable in some cell lines but not in others (30). It is tempting to speculate that the function of ICP22 late in infection is to enhance the expression of a specific subset of γ genes, possibly by specific interaction with a transcriptional factor.

(iii) The functions of the nuclear structures described in this and a preceding report (23) remain key questions. The compartment described in this report is clearly distinct from the ND10 domains described by Maul et al. (25), inasmuch as the ND10 domains are disrupted by the action of ICP0 following infection with HSV (11). The term "replication compartment" has been given to nuclear structures reported to contain viral DNA synthesis proteins and especially ICP8, a single-stranded DNA binding protein (7). The designation "replication compartment" given to it by Quinlan et al. (34) is unfortunate for several reasons. (a) The entire cell is a replication compartment, and viral replication cannot continue without either nucleus or cytoplasm. (b) Viral DNA synthesis begins between 3 and 6 h after infection (39), i.e., several hours before the formation of the structures that we observed. (c) The mere presence of ICP8 is not an indication that DNA synthesis is the exclusive, the predominant, or even a required activity of replication compartments, particularly since ICP8 has also been reported to play a role in regulation of viral gene expression. Godowski and Knipe (13) reported that ICP8 blocks expression of γ_2 genes located in the genomes of input, parental virus. Precisely how this is accomplished, however, is not clear since the same laboratory (18) showed that in the presence of inhibitors of viral DNA synthesis, ICP8 is dispersed and no longer colocalizes with the replication compartment. Also, inhibition of viral DNA synthesis does not preclude the transcription of at least γ_1 genes. (d) Several laboratories have reported colocalization of ICP4 and RNA pol II with the

replication compartment (18, 35, 36). As we and others noted, colocalization requires onset of viral DNA synthesis. All indications, however, are that the colocalization of DNA synthesis with these factors does not imply a continuous functional interaction. ICP4 does not bind to single-stranded DNA, and moreover, Knipe et al. (18) reported that in the presence of inhibitors of viral DNA synthesis, ICP4 is retained in this compartment whereas ICP8 is not. As these authors noted, "the association of ICP4 was independent of viral DNA synthesis once the compartments were formed."

Operationally, the question is whether transcription occurs simultaneously with viral DNA synthesis on the same molecule or whether the two processes occur on different molecules. Taken together, the data support the hypothesis that transcription of late genes can occur in the absence of ICP8 and therefore in the absence of DNA synthesis. It follows then that viral DNA synthesis required for transcription of late genes occurs in a spatially confined space but on different sequences and in perhaps entirely different macromolecular structures. The designation replication compartment, apart from being a misnomer, mutes the difference in the biochemical events taking place in two overlapping compartments.

The localization of viral transcriptional factors to specific structures is of considerable significance. First and foremost, this report places the molecular analysis of the shift from α and β to γ gene transcription on a plane much more amenable to investigation, as the content of these structures can be investigated in situ by appropriate probes. An important contribution to this approach is the evidence that ICP22 may in fact be a transcriptional factor.

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