Small Dense Nuclear Bodies Are the Site of Localization of Herpes Simplex Virus 1 U_L3 and U_L4 Proteins and of ICP22 Only When the Latter Protein Is Present

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The herpes simplex virus 1 U_L3 and U_L4 open reading frames are expressed late in infection and are not essential for viral replication in cultured cells in vitro. An earlier report showed that the U_L4 protein colocalizes with the products of the $\alpha22/U_S1.5$ genes in small nuclear dense bodies. Here we report that the U_L3 protein also colocalized in these small nuclear dense bodies and the localization of U_L3 and U_L4 proteins in these bodies required the presence of $\alpha22/U_S1.5$ genes. In cells infected with a mutant lacking intact $\alpha22/U_S1.5$ genes, U_L3 was diffused throughout the nucleus even though the overall accumulation of the $\gamma2$ U_L3 protein was decreased. The results suggest that ICP22 acts both as a regulator of U_L3 accumulation and as the structural component and anchor of these small dense nuclear bodies.

Of the 84 open reading frames of herpes simplex virus 1 (HSV-1) known to be expressed in infected cells, more than half are dispensable for viral replication at least in some cell lines maintained in culture (18, 19). These open reading frames appear to be essential for viral survival in nature, since viruses lacking these genes have not been isolated and in experimental animal systems mutants lacking these genes tend to be attenuated. In many instances the functions of these genes are difficult to study, since they appear to have no obvious phenotype in infected cells. This report is an extension of recently published studies on two such genes, U_L3 and U_L4 (8, 12).

Both U_L3 and U_L4 are dispensable in all cell lines tested to date (3; N. S. Markovitz and B. Roizman, unpublished data). Neither gene appears to play a role in pathogenicity or latent infections caused by HSV-1 (9; J. D. Baines and B. Roizman, unpublished data). The U_L3 gene products are expressed as multiple isoforms: two isoforms result from alternate translation initiation of the U_L3 open reading frame; the phosphoisoforms result from posttranslational processing by U_L13 viral protein kinase and by at least one additional, as yet unidentified protein kinase (7, 12). The HSV-2 U_L3 phosphoprotein is present in multiple isoforms and localizes in the nucleus after the onset of DNA synthesis (22). Both U_L3 and U_L4 are late, or $\gamma2$ proteins whose synthesis depends on the onset of viral DNA replication. HSV-1 and HSV-2 U_L4 proteins have been described elsewhere (5, 8, 23). Recently this laboratory reported that U_L4 colocalizes with the products of the $\alpha22/U_S1.5$ genes in the nucleus at 17 h after infection (8).

The $\alpha22/U_S1.5$ gene is located at the left terminus of the unique short sequence (U_S) when the genome is presented in its prototype orientation (4, 15, 21). $\alpha22$ encodes the 420-amino-acid ICP22. The $U_S1.5$ protein is expressed from a shorter mRNA and consists of approximately 60% of carboxyl-terminal amino acid sequence of the ICP22 (4). The shared portion of the $\alpha22/U_S1.5$ gene products is required for efficient viral replication in rodent and rabbit cell lines (14), and its

presence enhances the expression of a subset of $\gamma2$ genes (14, 16, 17, 20). Although we refer to ICP22 in the colocalization studies, our studies do not differentiate between ICP22 and the $U_S1.5$ protein.

The studies described in this report utilized the F strain of HSV-1 [HSV-1(F)]. In the recombinant viruses R8105 and R4660, a sequence encoding an epitope reacting with an available monoclonal antibody was inserted in frame into the coding sequences of U_L3 and U_L4 genes, respectively (8, 12). The monoclonal antibody CH28-2 (Goodwin Cancer Research Institute, Plantation, Fla.) reacts with an epitope mapped to the glycoprotein B of human cytomegalovirus and does not react with HSV-1 proteins (11). In recombinant virus R325, the carboxyl-terminal 220 amino acids of the $\alpha22/U_S1.5$ gene had been deleted (15). In the recombinant R4968, the deletion in $\alpha22$ has been restored (13). The origin and maintenance of the HEp-2 and rabbit skin cells have been described elsewhere (8, 12). The antisera used in these studies have also been reported elsewhere. Briefly, rabbit antisera were raised against a chimeric protein containing amino acids 44 to 235 of the HSV-1 U_L3 protein and, separately, against the entire HSV-1 U_L4 protein (8, 12). Rabbit antiserum (R77) to ICP22 was made against the amino-terminal domain of ICP22 and does not react with the $U_S1.5$ protein (2). The monoclonal antibody to ICP4 (H640) described elsewhere (1) was purchased from Goodwin Cancer Research Institute.

In the first of a series of immunofluorescence studies (data not shown), we noted that the pattern of U_L3 fluorescence was similar to that of small dense nuclear bodies containing both ICP22 and U_L4 (8). Specifically, an earlier report showed that U_L4 protein colocalized with ICP22 in small dense nuclear structures (8). To verify this conclusion, rabbit skin cells grown in wells on glass slides were exposed to approximately 10 PFU of HSV-1(F) or of recombinant virus per cell and incubated at 37°C. The cells were fixed at 17 h after infection in methanol and processed as described elsewhere (8). The results were as follows.

(i) The U_L3 protein localized in small dense nuclear bodies (Fig. 1A to C). Furthermore, this experiment showed that identical images were obtained with antibody to the tag (fluorescein isothiocyanate [FITC]) and the authentic antibody to the U_L3 protein (Texas red). Thus, as was the case for U_L4 (8),

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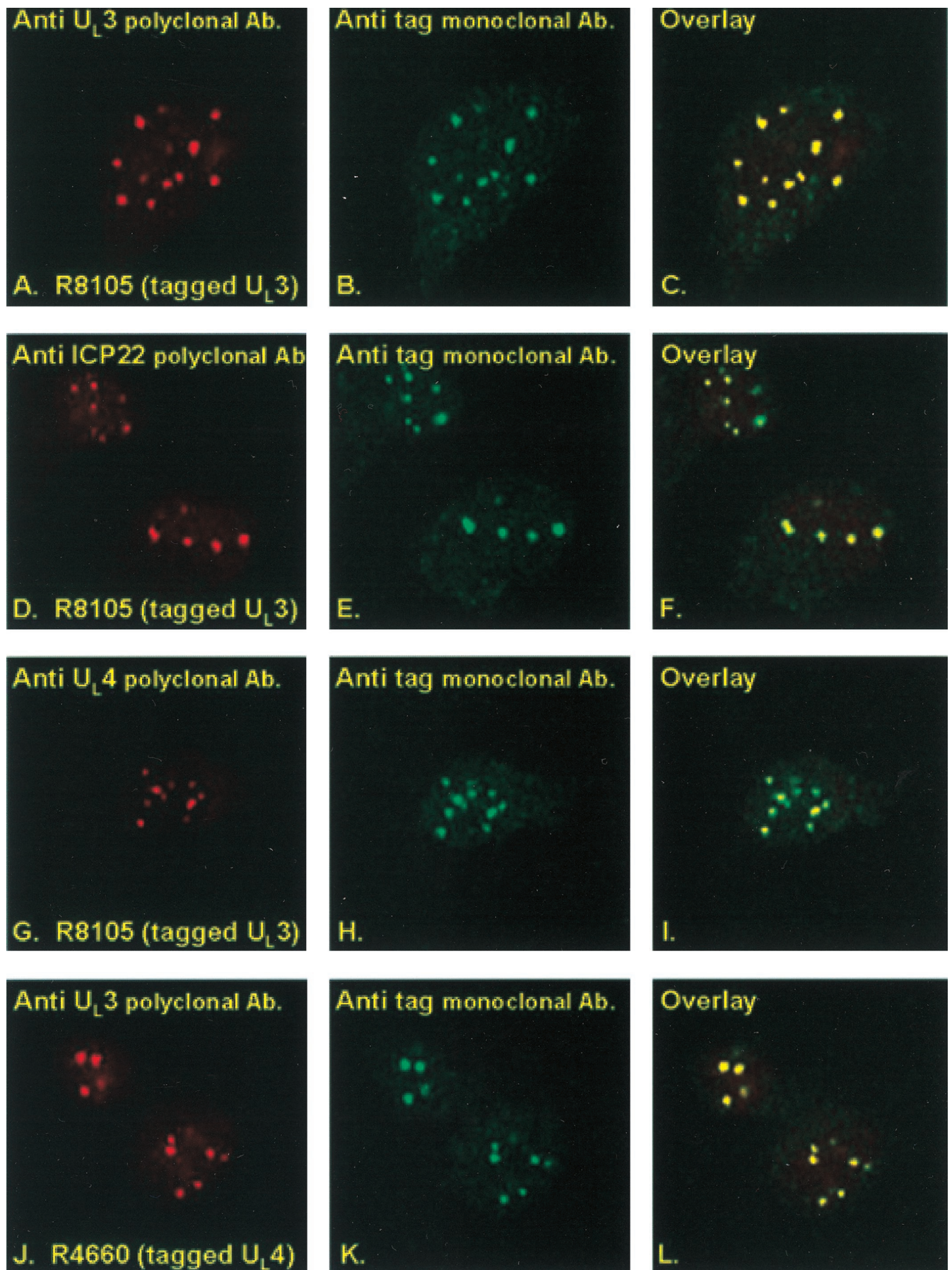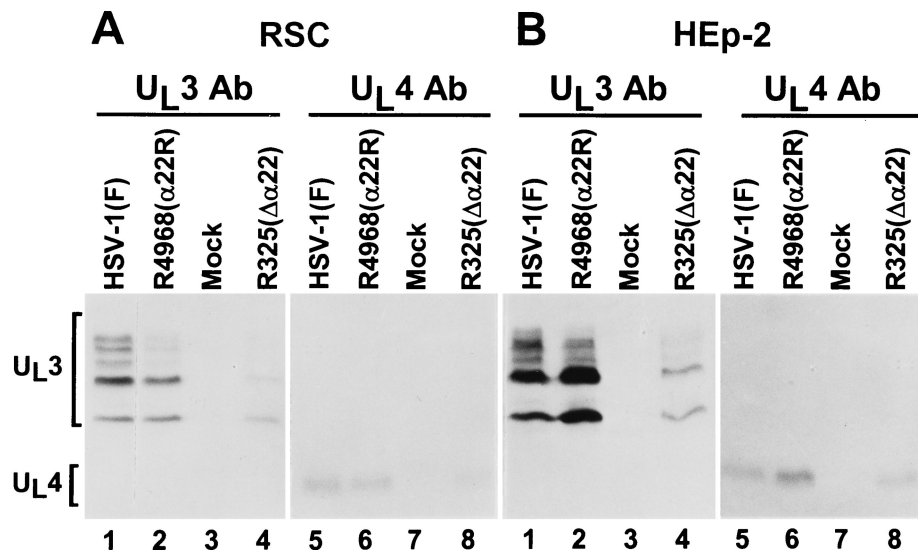
FIG. 2. Immunoblot of electrophoretically separated proteins from cell lysates of rabbit skin cells (RSC) (A) or HEp-2 cells (B) probed with antibody (Ab) to U_L3 (lanes 1 to 4) or U_L4 (lanes 5 to 8). Lysates of cells mock infected or infected with HSV-1(F) or the indicated recombinant virus were prepared as described elsewhere (12).

the virus carrying the tagged U_L3 could be used as a substitute for the untagged virus in double-label studies.

(ii) The U_L3 proteins colocalized with ICP22 in cells infected with R8105 (U_L3 epitope tagged) and reacted with the antibody against ICP22 (R77) and the anti-tag monoclonal antibody (Fig. 1D to F). In this instance the superimposition shown in Fig. 1F was not nearly as complete as that seen in Fig. 1C. It is noteworthy that in some of the dense bodies the U_L3 protein was either more diffuse or more abundant than ICP22.

(iii) The amount of U_L4 antigen was significantly lower than that of the U_L3 protein in cells infected with R8105 (U_L3 epitope tagged) and reacted with the rabbit polyclonal antibody to U_L4 and to the anti-tag monoclonal antibody. Nevertheless, the U_L3 and U_L4 proteins colocalized, especially in dense bodies in which the U_L4 protein was readily detectable (Fig. 1G to I). The reverse experiment yielded results consistent with that conclusion. Specifically, in cells infected with R4660 (tagged U_L4) and reacted with the polyclonal rabbit antibody to U_L3 and the anti-tag monoclonal antibody, the signal for the tagged U_L4 protein was stronger than that obtained in Fig. 1G with the anti-U_L4 antibody. In this instance the two images produced by the two antibodies were superimposable.

(iv) We have selected rabbit skin cells for these studies on the basis of the observation that the nuclear structures containing U_L3 and U_L4 were larger or more readily detectable. Studies on infected HEp-2 cells yielded results identical to those described above (data not shown).

In earlier reports we described the properties of the antibodies to the U_L3, U_L4, and ICP22 proteins (2, 8, 12). Relevant to the studies described here, the antibodies made against U_L3 and U_L4 are specific for each protein and do not cross-

react. In both immunofluorescence and immunoblot assays, U_L3 and U_L4 proteins were not visualized when the anti-U_L4 and U_L3 antibodies, respectively, were used (Fig. 2) (8, 12), and U_L3 and U_L4 do not have protein sequence homology. We conclude from these studies that ICP22, U_L3, and U_L4 proteins colocalize in nuclear structures of infected cells.

The second series of experiments was prompted by earlier observations that ICP22 enhances the expression of a subset of γ2 proteins and that modification of the phosphoprotein isoforms of U_L3 is mediated by the viral U_L13 protein kinase. In numerous studies, this laboratory has found a link between U_L13 protein kinase and ICP22 (14, 16, 17, 20). In every case examined, the phenotype of U_L13^- mutants was similar to that of $α22^-/U_S1.5^-$ mutants (13, 14, 16, 17). To unravel the effect of ICP22 on the accumulation of U_L3 and U_L4, electrophoretically separated lysates of mock-infected cells or cells infected with HSV-1(F), R325 ($Δα22/U_S1.5$), or the repair virus R4968 were transferred to nitrocellulose sheets and probed with antibodies to either U_L3 or U_L4. The results of this experiment shown in Fig. 2 indicate that, consistent with earlier results (Markovitz and Roizman, unpublished), in cells infected with the R325 ($Δα22/U_S1.5$) virus, all isoforms of U_L3 protein were present but in smaller amounts than in cells infected with the wild-type virus or with the recombinant virus in which the α22 gene had been repaired (R4968) (Fig. 2, lanes 1 to 4), whereas the levels of U_L4 protein showed a less dramatic decrease (Fig. 2, lanes 5 to 8).

Since U_L4 and U_L3 proteins colocalize with ICP22 and deletion of ICP22 resulted in a decreased accumulation of U_L3 and, to a lesser extent, of U_L4 proteins, the question arose as to whether the localization of these proteins was affected in cells infected with the recombinant virus R325 from which the

FIG. 1. Colocalization of ICP22 and U_L4 protein with U_L3 protein. Rabbit skin cells were exposed to HSV-1 recombinant virus R8105 (A to I) or R4660 (J to L) at a ratio of 10 PFU/cell. At 17 h after infection, the cells were fixed with cold ethanol and reacted with the indicated antibodies. Primary antibodies were diluted 1:500 to 1:1,000. Goat anti-rabbit immunoglobulin conjugated to Texas red (Molecular Probes, Eugene, Oreg.) was used to reveal the antigen reacting to the rabbit antisera against U_L3, U_L4, and ICP22, while anti-mouse immunoglobulin conjugated to FITC (Sigma) was used to visualize the mouse monoclonal antibody CH28-2. The slides were examined under a Zeiss confocal fluorescence microscope, and laser-scanned digitized images of the cells stained with fluorescent antibody were acquired with software provided with the Zeiss confocal microscope. Ab., antibody.
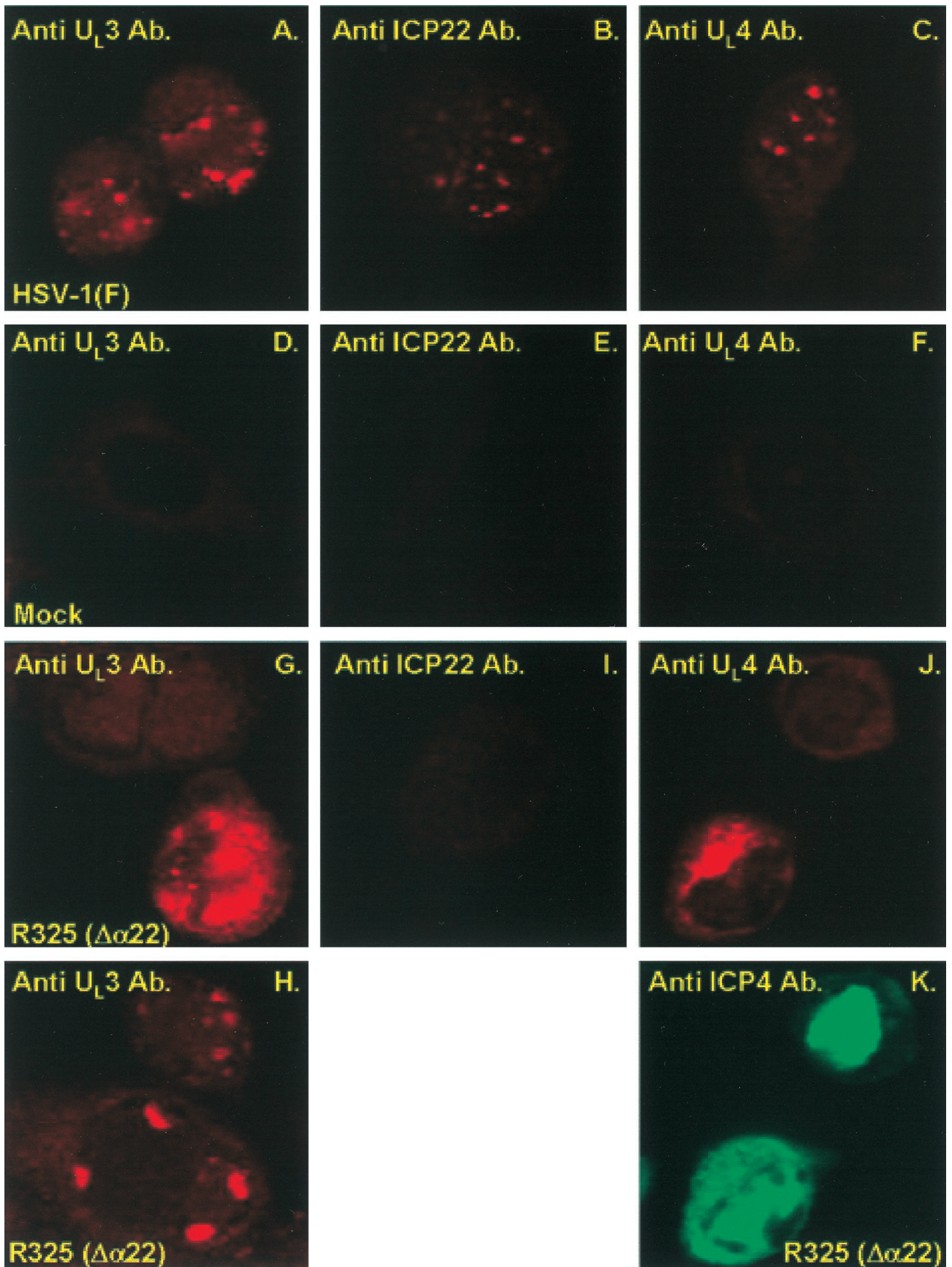
FIG. 3. Localization of U_L3 and U_L4 proteins and of ICP22 in infected cells. Rabbit skin cells were mock infected or exposed to 10 PFU of HSV-1(F) or R325 per cell and processed as described in the legend to Fig. 1. Anti-mouse immunoglobulin conjugated to FITC (Sigma) was used to visualize the mouse monoclonal antibody to ICP4 (K). Ab., antibody.

carboxyl-terminal 220 codons of the α22 gene had been deleted. In this series of experiments, rabbit skin cells were mock infected or infected with HSV-1(F) or recombinant R325 virus and reacted with single antibodies (Fig. 3A to I) or U_L4 and ICP4 antibodies (Fig. 3J and K). The results were as follows.

(i) As expected, neither U_L3, U_L4, or ICP22 antibodies reacted with mock-infected cells (Fig. 3D to F).

(ii) In HSV-1(F)-infected cells, polyclonal antibodies to U_L3, U_L4, and ICP22 reacted with their respective antigens in small dense nuclear structures (Fig. 3A to C).

(iii) In R325 mutant-infected cells, the expression patterns of U_L3 and U_L4 differed from that observed in HSV-1(F)-infected cells in two respects. First, we observed that fewer infected rabbit skin cells expressed detectable U_L3 protein—a finding consistent with the observation that U_L3 protein accumulated in smaller amounts in cells infected with R325 recombinant virus than in cells infected with wild-type virus. The fraction of cells expressing U_L4 was also decreased in R325-infected cells. To verify that the difference in the proportion of cells expressing U_L3 and U_L4 in R325-infected cells was related to relative U_L3 and U_L4 levels and not to a difference in infectivity, replicate wells were reacted with antibodies to the ICP4 protein. Figure 3J and K show the same two cells. While virtually all cells were infected, as evidenced by the presence of the ICP4 protein (Fig. 3K), only some cells were positive for U_L3 (data not shown) or U_L4 (Fig. 3J) protein expression. Thus, the microscopic fields shown in Fig. 3J and K are identical; only one of the two infected cells shown in Fig. 3K contains U_L4 protein. The second interesting finding was that in the cells positive for U_L3 protein, the distribution of U_L3 was more diffuse, formed irregularly shaped structures that occupied a significantly larger portion of the nucleus (Fig. 3G and H). A similar phenomenon was observed for U_L4 (Fig. 3J).

(iv) Studies on infected HEp-2 cells yielded results identical to those described above.

A curious phenomenon observed in these studies is that although U_L3 protein accumulated in reduced quantities in cells infected with α22$^-$/U_S1.5$^-$ virus-infected cells, the quantity of U_L3 protein as measured by immunofluorescence appeared to be greater in those cells expressing U_L3 than that observed in cells infected with wild-type virus. One likely explanation is that in a dispersed mode, as seen in cells infected with α22$^-$/U_S1.5$^-$ virus, the U_L3 protein was far more accessible to its antibody than in the compressed mode in the small, dense nuclear structures of cells infected with wild-type virus. We conclude from the studies described in this report the following.

(i) Both U_L3 and U_L4 are late or γ2 genes dependent on viral DNA synthesis for their expression. Efficient accumulation of U_L3 protein, nascent or posttranslationally modified, requires the expression of U_L13 protein kinase (12) and of the α22/U_S1.5 genes. In the absence of the α22/U_S1.5 genes, neither U_L3 nor U_L4 localizes in the small dense nuclear structures seen in cells infected with wild-type virus. ICP22 by itself in transfection assays forms small dense nuclear bodies (V. Galvan and B. Roizman, unpublished data). Thus, no late protein is required for the formation of the dense bodies, since ICP22 alone is sufficient. Furthermore, the U_L3 and U_L4 genes do not have appreciable protein sequence homology. These results indicate that colocalization of U_L3 and U_L4 is directed by ICP22 rather than by the interaction of U_L3 and U_L4 proteins.

(ii) The function of these small dense nuclear bodies is not known. Earlier in infection, ICP22 localized in structures resembling the small dense nuclear structures described here. After the onset of viral DNA, ICP22 colocalizes with ICP4, RNA polymerase II, and nascent DNA and occupies a much larger volume than the small dense nuclear structures seen very early or very late in infection do (10). Late in infection, ICP22 again localizes in small dense nuclear structures along with U_L3 and U_L4. The observation that ICP22 directs the localization of the newly synthesized U_L3 and U_L4 proteins to such structures suggests that they play a role in the viral replicative cycle late in infection.

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