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Cooperativity among Herpes Simplex Virus Type 1 Immediate-Early Regulatory Proteins: ICP4 and ICP27 Affect the Intracellular Localization of ICP0

ZHIMIN ZHU, WEIZHONG CAI,† AND PRISCILLA A. SCHAFFER*

Division of Molecular Genetics, Dana-Farber Cancer Institute, and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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The results of transient expression assays and studies of viral mutants have shown that three of the five immediate-early proteins of herpes simplex virus type 1 (HSV-1) perform regulatory functions, individually and cooperatively. As part of efforts designed to explore the molecular basis for the functional cooperativity among ICP0, ICP4, and ICP27 in the regulation of HSV gene expression, we have examined the intracellular localization of ICP0 in cells infected with ICP4 and ICP27 null mutant viruses by indirect immunofluorescence. Although ICP0 was localized predominantly to the nuclei of wild-type virus-infected cells, it was found exclusively in the nuclei of ICP27 mutant-infected cells and in both the cytoplasm and nuclei of ICP4 mutant-infected cells, the cytoplasmic component being especially strong. These observations indicate that both ICP4 and ICP27 can affect the intracellular localization of ICP0. Transient expression assays with plasmids that express wild-type and mutant forms of ICP0, ICP4, and ICP27 confirmed that ICP4 promotes and that ICP27 inhibits the nuclear localization of ICP0. These results confirm the observations made for mutant virus-infected cells and indicate that the localization pattern seen in infected cells can be established by these three immediate-early proteins exclusive of other viral proteins. The C-terminal half of ICP27 was shown to be required to achieve its inhibitory effect on the nuclear localization of ICP0. The region of ICP0 responsive to ICP27 was mapped to the C terminus of the molecule between amino acid residues 720 and 769. In addition, the concentration of ICP27 was shown to have a significant effect on the intracellular localization of ICP0. Because the major regulatory activities of ICP0, ICP4, and ICP27 are expressed in the nucleus, the ability of these three proteins collectively to determine their own localization patterns within cells adds a new dimension to the complex process of viral gene regulation in HSV.

Herpes simplex virus (HSV) gene expression has long been known to proceed in a sequential and highly regulated manner (31). Upon entry of viral DNA into the nucleus, five viral genes encoding infected cell polypeptides (ICP0, ICP4, ICP22, ICP27, and ICP47) are transcribed and translated by cellular machinery (10). Expression of these five immediate-early (IE) viral proteins is activated by VP16, a protein present in infecting virus particles (4, 8), and does not require prior viral protein synthesis (31, 37). The five IE proteins are the major regulatory proteins of the virus and are required to mediate the expression of early (E) and late (L) genes. The protein products of E genes are involved in viral DNA replication. Once DNA replication has begun, L genes are expressed, most likely from progeny viral DNA molecules. The protein products of L genes are the building blocks of new virus particles.

The manner in which the HSV regulatory cascade is orchestrated by IE proteins is unclear; however, much is known about the functions of three of the five IE proteins (ICP0, ICP4, and ICP27) from transient expression (chloramphenicol acetyltransferase) assays and from studies of viral mutants unable to express these proteins (6, 15, 17, 39, 57, 58, 65). ICP4 is the major transcriptional regulatory protein of the virus. It is required to activate transcription of E and L genes and to repress its own transcription as well as that of the other IE genes (13, 14, 17, 47). Although it is not a classic transcriptional activator, ICP0 is able to enhance the expression of any viral gene that exhibits a basal level of transcription (42). Because IE and E genes are transcribed at basal levels by the cellular transcription machinery, ICP0 serves to further enhance their expression. In transient assays, ICP27 by itself appears to have little effect on the expression of HSV genes (20, 60); however, it is able to further enhance or repress the transactivating activities of ICP4 and ICP0 (20, 39, 50, 60, 66).

Studies of viral mutants that lack the genes encoding ICP0, ICP4, and ICP27 have shown that ICP4 and ICP27, but not ICP0, are essential for virus replication (13, 38, 58, 65). Although ICP0 is not absolutely required for replication, it is necessary for efficient expression of E and L genes—especially at low multiplicities of infection—and thereby confers an advantage on the ability of the virus to replicate at low multiplicities (7, 22, 65).

Several lines of evidence suggest that ICP0, ICP4, and ICP27 act cooperatively. First, in transient expression assays, ICP0 and ICP4 have a synergistic enhancing effect on E and L gene expression, whereas ICP27 can exert both positive and negative effects on ICP0- and ICP4-induced gene expression, as noted above (19, 20, 23, 39, 45, 49, 51, 60). Thus, ICP27 represses IE gene expression induced by ICP0 and E gene expression induced by ICP0 and ICP4, whereas it further enhances L gene expression induced by ICP0 and ICP4. Second, the enhancing activity of ICP0 requires functional ICP4 during viral infection, but not in transient expression assays (16). Thus, despite the strong and broad transactivating activity of ICP0 by itself in transient assays, ICP0 exhibits no detectable enhancing activity in cells infected with ICP4 null

* Corresponding author. Phone: (617) 375-8240. Fax: (617) 375-8255. Electronic mail address: Priscilla_Schaffer@DFCI.Harvard.Edu.
† Present address: Oncogene Science, Inc., Uniondale, NY 11553-3649.

mutants, in which ICP0 is abundant (16). Third, studies of ICP27 mutant-infected cells and cells transfected with ICP27-expressing plasmids indicate that ICP27 is responsible, at least in part, for the posttranslational modification of ICP4 (39, 50, 66). ICP4 migrates faster in wild-type virus-infected cells and in cells cotransfected with ICP4- and ICP27-expressing plasmids than in cells infected with ICP27 null mutants or cells transfected with only an ICP4-expressing plasmid (39, 50, 66). Finally, Knipe and Smith have reported that in cells infected with the ICP4 temperature-sensitive (*ts*) mutant, *ts*756, at the nonpermissive temperature, ICP0 and ICP4 were found almost exclusively in the cytoplasm, whereas in wild-type virus-infected cells, both proteins were found predominantly in the nucleus (35). These authors hypothesized that the mutant ICP4 protein inhibited the nuclear localization of ICP0. One explanation proposed for this hypothesis was that the mutant form of ICP4 interacts with ICP0, retaining it in the cytoplasm.

Collectively, these observations suggest the possibility that either direct or indirect interactions occur among the three IE regulatory proteins. To determine whether the functional cooperativity among ICP0, ICP4, and ICP27 is reflected in the intracellular localization of the three proteins, we have used indirect immunofluorescence (IF) to examine the manner in which the localization of ICP0 is affected by ICP4 and ICP27 in infected and transfected cells. Here, we report that ICP4 has an enhancing effect and that ICP27 has an inhibitory effect on the nuclear localization of ICP0 and that the level of ICP27 expressed is critical for its inhibitory effect.

MATERIALS AND METHODS

Cells and viruses. All infections and transfections were performed with Vero cells. Vero cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as previously described (57). HSV type 1 (HSV-1) strain KOS was used as the wild-type virus, and all mutants used in the study were derived from KOS. The ICP0 mutant viruses (7134, n428, n525, n680, n720, and n770) (6) and the ICP22 mutant virus (22/n199) (2) were propagated and assayed in Vero cells as described previously (59). The ICP27 deletion mutant virus, 5dl1.2 (38), and the ICP4 nonsense mutant virus, n12 (16), were propagated and assayed on 3-3 and E5 cells, respectively (15, 38). 3-3 and E5 cells are Vero cells stably transformed with the genes for ICP27 and ICP4, respectively.

Plasmids. The plasmids used in transfections include the following. (i) Plasmid pSH contained the wild-type ICP0 gene, and pSH-derived ICP0 nonsense mutant plasmids were pn428, pn525, pn680, pn720, and pn770 (6). These plasmids contained the same mutations as those in mutant viruses n428, n525, n680, n720, and n770. The number designations of ICP0 nonsense mutant plasmids and viruses indicate the codons into which the nonsense linker was inserted to generate individual mutations (6). For example, n428 contains a nonsense linker in codon 428 of the 775-amino-acid ICP0 protein. (ii) Plasmid pKHX-BH contained the wild-type ICP27 gene and pKHX-BH-derived ICP27 nonsense mutant plasmids (n6, n7, n8, n9, and n11) and deletion mutant plasmids (d1, d2, and d3) (39). (iii) Plasmid pSG130B/S contained the wild-type ICP27 gene and pSG130B/S-derived ICP27 insertion or deletion mutant plasmids (pN9M, pR3IF, pS5, pN6, pF21, pS13, pS1B, and pS18) kindly provided by R. M. Sandri-Goldin (University of California, Irvine) (30). (iv) Plasmid pn11 contained the wild-type ICP4 gene, and plasmid pn3 contained a mutant ICP4 gene which encodes only the N-terminal 251 amino acids of the intact ICP4 molecule. Plasmid pn3 contained the same mutation as that in mutant virus n12 (15, 16).

Antibodies. Mouse monoclonal antibodies H1083 and H1113, specific for ICP0 and ICP27, respectively (1), were kindly provided by L. Pereira (University of California, San Francisco). Monoclonal antibody (MAb) 58S is specific for ICP4 (61). Rabbit polyclonal antibody J17, specific for ICP0, was produced in this laboratory. Rabbit polyclonal antibody R021, which is directed against ICP4 (11), was kindly provided by R. Courtney (Pennsylvania State University College of Medicine, Hershey, Pa.). Rhodamine isothiocyanate (RITC)-conjugated goat anti-mouse, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin antibodies were obtained from Cappel Laboratories (Durham, N.C.).

Transfection. The calcium phosphate coprecipitation method of transfection was used in all tests (9). Briefly, 24 h prior to transfection, 5×10^5 Vero cells were seeded in 60-mm petri dishes with or without sterile 18-mm circular glass coverslips for use in IF tests and immunoblot analysis, respectively. Three hours prior to transfection, the medium was changed. For transfection, 10 μg of total DNA (plasmid DNA plus salmon testis carrier DNA) was suspended in 250 μl of 2× BES transfection buffer {50 mM BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid], 280 mM NaCl, 1.5 mM Na_2HPO_4 [pH 6.95]}. The DNA solution was mixed with an equal volume of 250 mM $CaCl_2$ for 30 min. At 24 h postseeding, the resulting suspension containing the DNA precipitate was added to the medium that overlaid cell monolayers, and transfected cultures were incubated at 37°C. For the IF tests shown in Fig. 2a and b, 3, and 4, the medium was removed after 4 h of incubation and the cells were washed with TBS (137 mM NaCl, 5 mM KCl, 25 mM Tris-HCl [pH 7.4]) and treated with 15% glycerol (in 1× BES transfection buffer) for 2 min. The cells were then washed twice with TBS, fresh medium was added, and cultures were incubated at 37°C for an additional 20 h before processing for IF. For immunoblots (see Fig. 2c and 8), transfected cells were not treated with glycerol but were incubated continuously at 37°C for 20 h and then washed with TBS and refed with fresh medium for an additional 4 h at 37°C. These two procedures produced similar results; however, transfection by the latter procedure was more efficient (reference 9 and this study).

Immunoblot analysis. At 24 h posttransfection, the cells were washed with TBS and lysed with lysis buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40) containing 1 mM leupeptin (Sigma, St. Louis, Mo.) and 5% dry milk to inhibit proteinase activities present in Vero cell extracts. Cell extracts were then subjected to immunoprecipitation in the following manner. Extracts were incubated with H1083 (1:2,000) for 60 min on ice and then with freshly prepared rabbit anti-mouse IgG-bound Pansorbin cells (Calbiochem, San Diego, Calif.) for 30 min on ice. The ICP0-H1083 complex was concentrated by microcentrifugation and washed with lysis buffer before dissociation in sample buffer (50 mM Tris-HCl [pH 7.0], 2% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 5 μg of bromophenol blue per ml). Precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (36) as modified by Gibson et al. (26). The separated proteins were transferred to a nitrocellulose filter by electroblotting (5, 67). ICP0 was detected by treating the filter with mouse MAb H1083 (1:2,000 dilution) and then by an alkaline phosphatase detection system (Promega Biotec, Madison, Wis.).

IF tests. Vero cells were grown on coverslips as described above. At 24 h postseeding, cells were infected with wild-type or mutant viruses at a multiplicity of 20 PFU per cell or less, as

indicated in Fig. 6 and 7, or were transfected by the calcium-phosphate coprecipitation procedure with plasmid DNAs in the combinations and the concentrations described in the text. Infected monolayers on coverslips were processed for IF at 5 h postinfection, and transfected monolayers were processed at 24 h posttransfection.

The indirect IF procedure used in this study was performed as follows (28). Cells on coverslips were fixed in 3.7% formaldehyde for 30 min, permeabilized for 2 min in acetone at −20°C, immediately rehydrated in sterile water, and equilibrated in phosphate buffered saline (PBS). Fifty microliters of primary antibody diluted in PBS (H1083 [1:800], 58S [1:50], R021 [1:150], H1113 [1:600], and J17 [1:50]) was added to monolayers on coverslips which were incubated for 30 min at 37°C in a humid chamber. After incubation, coverslips were washed with PBS, a secondary antibody conjugated to either FITC (1:200) or RITC (1:300) was added, and incubation was continued for 30 min at 37°C. The coverslips were then washed with PBS and water and mounted on glass slides with mounting medium containing 30 mM propyl gallate in glycerol gelatin (Sigma) to prevent quenching of the fluorescence. In Fig. 7a and 8, the cells were double stained simultaneously by using a mixture of the anti-ICP0 antibody, J17 (1:50), and the anti-ICP27 antibody, H1113 (1:600).

Monolayers were viewed with a Zeiss fluorescence microscope with a 40X/1.3, OIL, PH3, Plan-Neofluar objective. For detection of fluorescence from FITC-conjugated antibodies, a filter set (designated Fl) consisting of an exciter filter for light with wavelengths of 450 to 490 nm, a beam splitter for light with wavelengths less than 510 nm, and a barrier filter for light with wavelengths of from 512 to 565 nm was used. For detection of RITC fluorescence, a filter set (designated Rh) consisting of an exciter filter for wavelengths of 546 nm, a beam splitter for wavelengths less than 580 nm, and a barrier filter for wavelengths greater than 590 nm was used. Control experiments revealed complete exclusion of RITC fluorescence with the Fl filter set and of FITC fluorescence with the Rh filter set (see Fig. 7b). Photographs were taken with Kodak Tri-X Pan 400 film (black and white) or Kodak Gold 400 film (color).

RESULTS

Intracellular localization of ICP0, ICP4, and ICP27 in wild-type and mutant virus-infected cells. To determine whether IE proteins are able to affect their own intracellular localization, we examined the localization of ICP0, ICP4, and ICP27 in cells infected for 5 h with 20 PFU of KOS (wild-type) per cell or with viruses containing mutations in the genes encoding ICP0, ICP4, and ICP27 by indirect IF (Fig. 1).

A combination of diffuse and punctate ICP0-specific fluorescence was detected predominantly in the nuclei of KOS-infected cells, although both diffuse and punctate staining was also evident in the cytoplasm of some cells (Fig. 1A). By contrast, punctate ICP0-specific fluorescence was detected predominantly in the cytoplasm of cells infected with the ICP4 nonsense mutant virus, n12 (Fig. 1B), and punctate ICP0-specific fluorescence was detected exclusively in the nuclei of cells infected with the ICP27 deletion mutant virus, 5dl1.2 (Fig. 1C). The size of punctate ICP0-staining dots was somewhat heterogeneous in n12-infected cells but was relatively homogeneous in 5dl1.2-infected cells. No ICP0-specific fluorescence was detected in cells infected with the ICP0 null mutant virus, 7134 (Fig. 1D), or in mock-infected cells (Fig. 1E).

The location and pattern of ICP4-specific fluorescence were similar in cells infected with 7134 or KOS (Fig. 1F and I), in that staining was most prominent in globular replication compartments in nuclei as previously described by Knipe et al. (34), with a low level of diffuse ICP4-specific fluorescence in the cytoplasm, consistent with the findings of Yao et al. (74). Like ICP0, ICP4 was localized exclusively within nuclei of 5dl1.2-infected cells (Fig. 1H), although the staining pattern of ICP4 was more diffuse in these cells, in contrast to the punctate staining pattern of ICP0 in 5dl1.2-infected cells (Fig. 1C). The truncated ICP4 peptide specified by mutant virus n12, which comprises only the amino-terminal 251 amino acids of the intact 1,298-amino-acid ICP4 molecule and lacks the nuclear localization signal, did not react detectably with the ICP4-specific monoclonal antibody, 58S (Fig. 1G). However, the n12 polypeptide reacts strongly with the polyclonal rabbit antiserum, R021 (16). By using R021 antiserum, the n12 peptide was detected as diffuse staining predominantly in the cytoplasm of n12-infected cells (data not shown).

ICP27 was evident as diffuse staining predominantly in the nuclei and to a lesser extent in the cytoplasm of KOS-, n12-, and 7134-infected cells (Fig. 1K, L, and N, respectively). If one examines these cells carefully, however, the diffuse ICP27-specific signal in the cytoplasm was stronger in cells infected with n12 (Fig. 1L) than in those infected with KOS or 7134 (Fig. 1K and N).

An ICP22 nonsense mutant, 22/n199 (2), was also tested in this study. In 22/n199-infected cells, the staining patterns of ICP0, ICP4, and ICP27 were similar to those observed in KOS-infected cells (data not shown).

These observations demonstrate that the intracellular localization of ICP0 characteristic of wild-type virus-infected cells is altered in the absence of ICP4 or ICP27 but not ICP22.

The effects of ICP4 and ICP27 on the intracellular localization of ICP0 in transient expression assays. In light of the observations described above, we next asked whether in transient assays, ICP4 and ICP27 are able to affect the localization of ICP0 in the absence of other HSV-specified gene products. In these experiments, Vero cells were transfected with a plasmid expressing wild-type ICP0 (pSH) alone or together with a plasmid expressing either wild-type ICP4 (pn11), a truncated form of ICP4 (pn3), wild-type ICP27 (pKHX-BH), or a truncated form of ICP27 (n9). In these tests, the polyclonal rabbit anti-ICP0 serum, J17, was used as the primary antibody, although similar results were obtained with MAb H1083. Cells exhibiting fluorescence were counted and placed in one of three categories: cells that exhibited exclusively nuclear staining (N), cells that exhibited exclusively cytoplasmic staining (C), or cells that exhibited both nuclear and cytoplasmic staining (N+C). Examples of cells included in each of these categories are shown in Fig. 2a. Figure 2b shows the percentage of ICP0-positive cells that fell into each category following transfection with the indicated plasmid or plasmids. The number of ICP0-positive cells examined was 200 or greater for each transfection, and each transfection was repeated twice with identical results.

In cells transfected with a small amount (1 μg) of the ICP0-expressing plasmid, pSH, ICP0 was localized almost exclusively (90%) within the nuclei of IF-positive cells (Fig. 2b, panel A). In cells transfected with a larger amount (5 μg) of pSH, however, the percentage of cells that exhibited exclusively nuclear staining was reduced to approximately 50%, and the number of cells that exhibited both nuclear and cytoplasmic staining increased proportionally (Fig. 2b, panel E). In cells transfected with even greater quantities (10 μg) of pSH, the percentage of cells that exhibited exclusively nuclear staining was further reduced (33%), and the percentage of cells that exhibited both nuclear and cytoplasmic staining again increased (data not shown). Whatever the concentration of

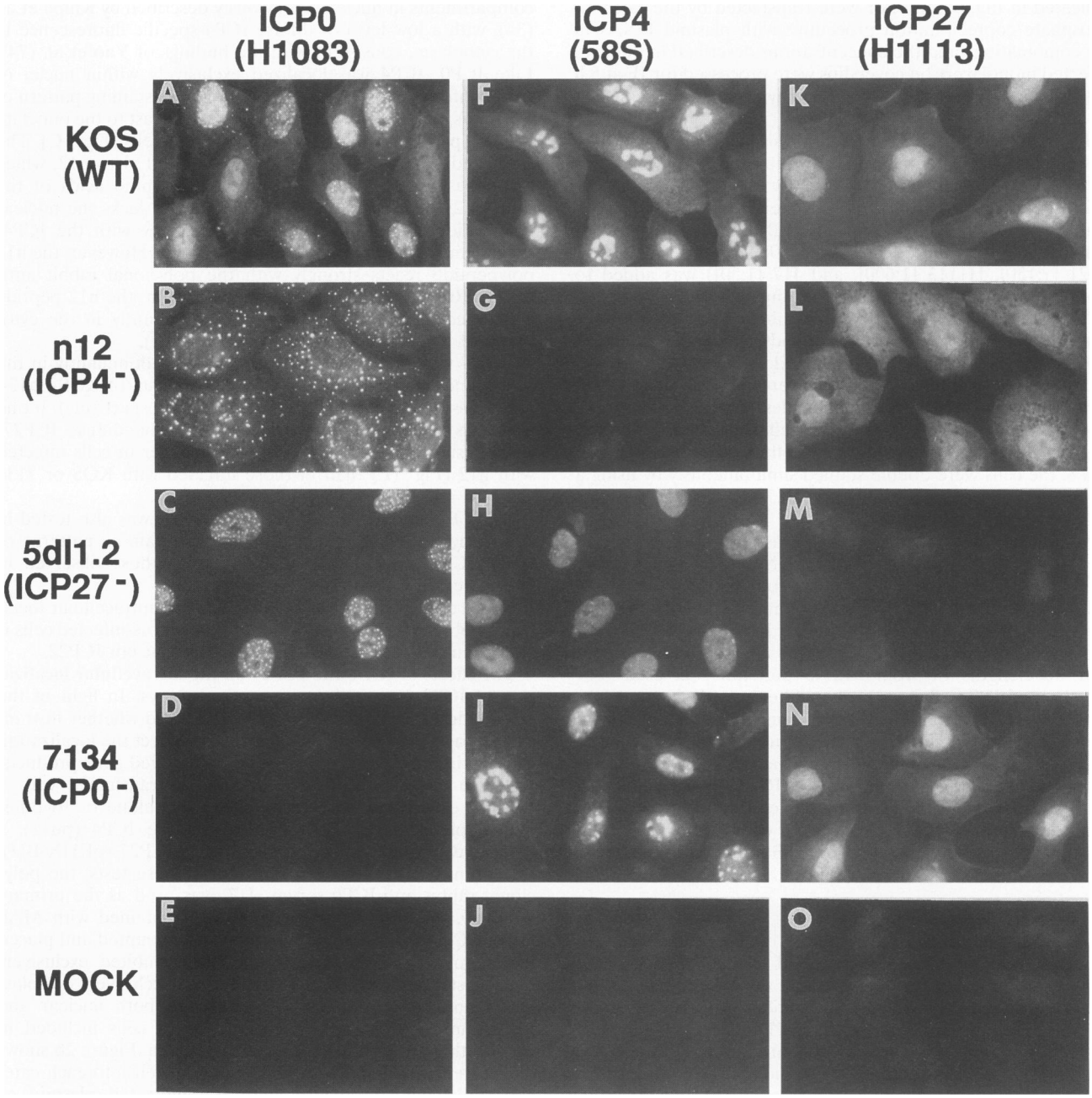


FIG. 1. Localization of ICP0, ICP4, and ICP27 in KOS- and mutant virus-infected cells. Vero cells were infected with 20 PFU of KOS per cell or with null mutants in ICP4 (n12), ICP27 (5dl1.2), and ICP0 (7134) or were mock infected at 37°C. At 5 h postinfection, monolayers on coverslips were fixed, permeabilized, and incubated with either H1083 mouse anti-ICP0 MAb, 58S mouse anti-ICP4 MAb, or H1113 mouse anti-ICP27 MAb as primary antibodies. RITC-conjugated goat anti-mouse immunoglobulin antibodies were used as the secondary antibody. The viruses used to infect cells are listed at the left of the micrograph, and the antibodies used in each test are listed at the top of the figure.

pSH that was used in transfections, however, cells expressing ICP0 exclusively in the cytoplasm or cells expressing ICP0 in the cytoplasm to a greater extent than in the nucleus were never observed.

In cells cotransfected with pSH and pn11, which express wild-type ICP0 and ICP4, respectively, a greater number of cells exhibited ICP0-specific fluorescence exclusively in the nucleus than following transfection with pSH alone (Fig. 2 [compare panels A with B and E with F]). With small amounts of the two plasmids (1 μg of each), the enhancing effect of ICP4 on the nuclear localization of ICP0 was difficult to assess, since approximately 90% of IF-positive cells exhibited nuclear fluorescence even in the absence of ICP4 (compare panels A and B). The enhancing effect of ICP4 on the nuclear localization of ICP0 was more apparent in cells transfected with larger amounts (5 μg of each) of both plasmids (compare panels E and F). The ICP4 mutant plasmid, pn3, which specifies the same truncated peptide as the nonsense mutant virus n12, had

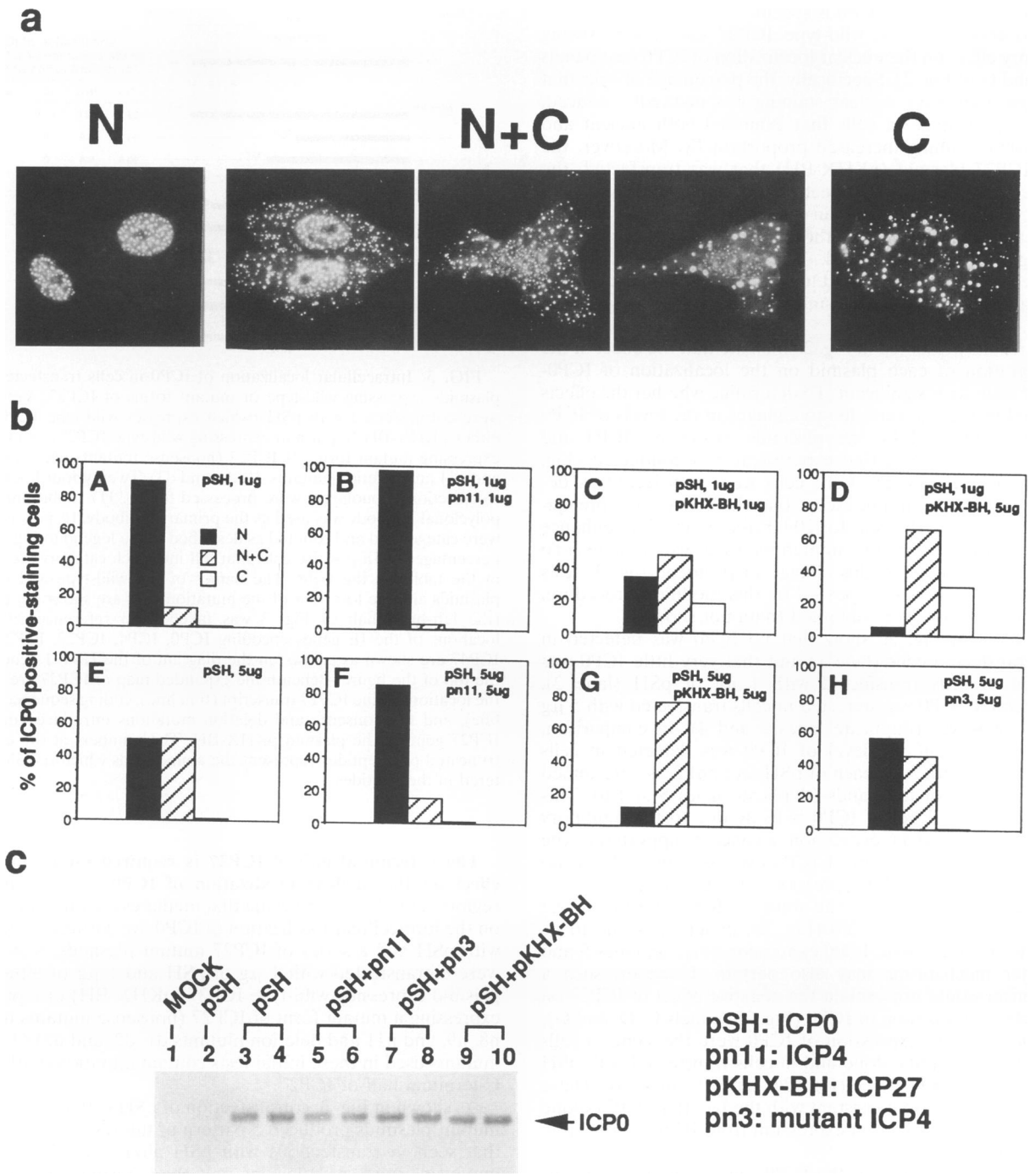


FIG. 2. Localization of ICP0 in cells transfected with plasmids expressing ICP0, ICP4, ICP27, or a mutant form of ICP4. Vero cells were transfected with pSH (which expresses wild-type ICP0) alone or together with either pn11 (which expresses wild-type ICP4) or pn3 (which expresses a form of mutant ICP4) or with pKHX-BH (which expresses wild-type ICP27) at the indicated concentrations. At 24 h after transfection, monolayers were processed for indirect IF by using rabbit anti-ICP0 polyclonal antibody J17. IF-positive cells were counted and categorized into one of three groups: cells that exhibited only nuclear staining (N), cells that exhibited only cytoplasmic staining (C), and cells that exhibited both nuclear and cytoplasmic staining (N+C). Examples of cells in each category are shown in panel a. The percentage of IF-positive cells that fell into each category is shown in panel b. The level of expression of ICP0 in transfected cells was also detected by immunoblotting with MAb H1083 as shown in panel c. Lanes: 1, mock-transfected cells; 2, cells transfected with 1 μg of pSH; 3 and 4, cells transfected with 5 μg of pSH; 5 and 6, cells transfected with 5 μg each of pSH and pn11; 7 and 8, cells transfected with 5 μg each of pSH and pn3; 9 and 10, cells transfected with 5 μg each of pSH and pKHX-BH.

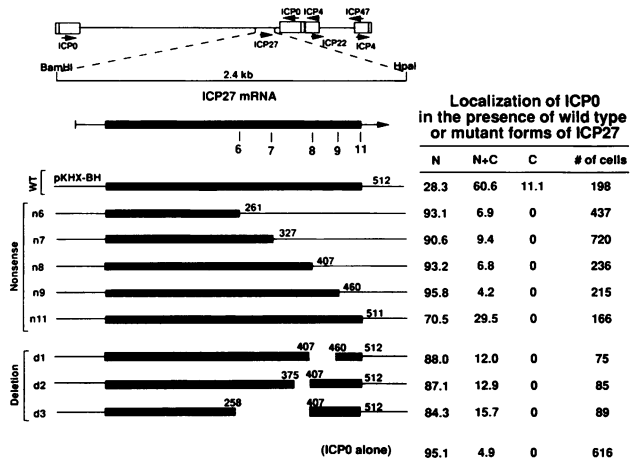
little effect on the localization of ICP0 (compare panels E, F, and H), demonstrating that the ability of ICP4 to promote the nuclear localization of ICP0 is specific.

In contrast to ICP4, wild-type ICP27 had a very strong inhibitory effect on the nuclear localization of ICP0 (see panels C, D, and G of Fig. 2). Specifically, the percentage of cells that exhibited exclusively nuclear staining was markedly reduced, and the percentage of cells that exhibited both nuclear and cytoplasmic staining increased proportionally. Moreover, the more ICP27 plasmid (pKHX-BH) that was transfected, the stronger was the negative effect (compare panels C and D of Fig. 2). Notably, it was only in tests involving ICP27 that the third category of cells, i.e., those that exhibited cytoplasmic staining only, was observed. The mutant form of ICP27 specified by plasmid n9 had no effect on the intracellular localization of ICP0-specific staining (not shown in Fig. 2; see Fig. 3).

The results presented in Fig. 2b indicate that the effect of the concentration of each plasmid on the localization of ICP0-specific staining is significant. To determine whether the effects observed in Fig. 2b were due to changes in the levels of ICP0 expression induced by the plasmids expressing ICP4 and ICP27, an immunoblotting experiment was conducted (Fig. 2c). In this experiment, Vero cells were transfected as described for Fig. 2a and b, except that the DNA-Ca^{2+} precipitates remained on the cells for 20 h rather than 4 h to enhance the yield of protein for immunoblotting experiments. To confirm the validity of this change in protocol, the IF tests shown in Fig. 2b were repeated by this modified procedure, and similar results were obtained (data not shown).

The blots in Fig. 2c show that no ICP0 was detected in mock-transfected cells (lane 1) and that very little ICP0 was detected in cells transfected with 1 μg of pSH (lane 2). Considerable ICP0 was detected in cells transfected with 5 μg of pSH, however (duplicate lanes 3 and 4). In comparison, approximately half the level of ICP0 was detected in cells cotransfected with 5 μg each of pSH and pn11, as determined by quantitation of the bands (duplicate lanes 5 and 6). This reduction in the level of ICP0 is likely due to the inhibitory effect of ICP4 on ICP0 expression, a concept supported by the fact that the mutant form of ICP4 expressed by pn3 had no inhibitory effect on ICP0 expression (duplicate lanes 7 and 8). Although it is possible that the ability of ICP4 to promote the nuclear localization of ICP0 (Fig. 2b, panel F) is due to its ability to down-regulate ICP0 expression (Fig. 2c, lanes 5 and 6), other mechanisms may also pertain. However, such a mechanism would not explain the negative effect of ICP27 on the nuclear localization of ICP0 (Fig. 2b, panels C, D, and G), since the levels of expression of ICP0 were the same in cells transfected with pSH alone and in cells transfected with pSH together with pKHX-BH (Fig. 2c, lanes 9 and 10). These observations are also consistent with the fact that ICP27 alone has no detectable effect on expression from IE promoters (39, 60), as noted above.

These data indicate that the ICP0-specific staining patterns observed in infected cells can be duplicated in transfections with ICP0 and ICP4 or with ICP0 and ICP27, indicating that other viral proteins are not required to establish these patterns. These data also demonstrate that ICP4, but not a mutant form of ICP4, has the ability to promote the nuclear localization of ICP0 in transient assays. This effect of ICP4 on ICP0 localization may be due in part to the ability of ICP4 to reduce the expression of ICP0. In contrast to ICP4, ICP27 (but not a mutant form of ICP27) has an inhibitory effect on the nuclear localization of ICP0 in transient assays; the more ICP27 plasmid that is transfected, the stronger is the negative effect.



Localization of ICP0 in the presence of wild type or mutant forms of ICP27

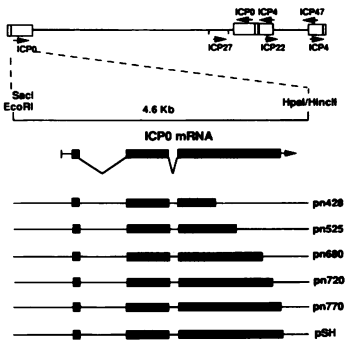
	N	N+C	C	# of cells
pKHX-BH	28.3	60.6	11.1	198
n6	93.1	6.9	0	437
n7	90.6	9.4	0	720
n8	93.2	6.8	0	236
n9	95.8	4.2	0	215
n11	70.5	29.5	0	166
d1	88.0	12.0	0	75
d2	87.1	12.9	0	85
d3	84.3	15.7	0	89
(ICP0 alone)	95.1	4.9	0	616

FIG. 3. Intracellular localization of ICP0 in cells transfected with plasmids expressing wild-type or mutant forms of ICP27. Vero cells were cotransfected with pSH (which expresses wild-type ICP0) and either pKHX-BH (a plasmid expressing wild-type ICP27) or plasmids expressing mutant forms of ICP27 (nonsense mutants n6, n7, n8, n9, and n11 and deletion mutants d1, d2, and d3). Twenty-four hours after transfection, monolayers were processed for IF. J17 rabbit anti-ICP0 polyclonal antibody was used as the primary antibody. IF-positive cells were categorized and counted as described in the legend to Fig. 2. The percentages of IF-positive cells that fell into each category are shown in the table on the right. The names of the wild-type and mutant plasmids and the locations of the mutation sites are shown on the left (the left-hand half of Fig. 3 was taken from reference 39). The locations of the IE genes encoding ICP0, ICP4, ICP22, ICP27, and ICP47 are shown as arrows on the diagram of the HSV-1 genome at the top of the figure. Beneath the expanded map of ICP27 are shown the locations of the ICP27 transcript (thin line), coding sequence (thick line), and the nonsense and deletion mutations introduced into the ICP27 gene in the plasmid pKHX-BH. The numbers at the ends of truncated polypeptides represent the amino acids which remain unaltered in the peptides.

The C-terminal half of ICP27 is required for its negative effect on the nuclear localization of ICP0. To identify the region of the ICP27 molecule that mediates its inhibitory effect on the intracellular localization of ICP0, we cotransfected cells with pSH and a series of ICP27 mutant plasmids. Vero cells were cotransfected with 1 μg of pSH and 1 μg of either the plasmid expressing wild-type ICP27 (pKHX-BH) or a plasmid expressing a mutant form of ICP27 (nonsense mutants n6, n7, n8, n9, and n11 and deletion mutants d1, d2, and d3) (39). All mutants used in these initial tests contain mutations within the C-terminal half of ICP27.

As shown in Fig. 3, cotransfection of pSH with each of these mutant plasmids produced a pattern of fluorescence similar to that seen in transfections with pSH alone. Notably, even a mutant plasmid (n11) lacking only the C-terminal amino acid residue exhibited a significantly reduced ability to block the nuclear localization of ICP0 relative to that of wild-type ICP27 (pKHX-BH). In n11, the terminal Phe is replaced by Leu, Ala, and Ser residues (39). On the basis of the mutational analysis shown in Fig. 3, no specific region within the C-terminal half of the molecule was shown to be responsible for the inhibitory effect of ICP27. Rather, the entire C-terminal half of the ICP27 molecule or the conformation of the molecule is required for its effect on the nuclear localization of ICP0.

In a further attempt to define the region of ICP27 required for its inhibitory effect, we tested a second series of KOS-



Localization of ICP0

	- ICP27				+ ICP27			
	N	N+C	C	#	N	N+C	C	#
pn428	0	100	0	17	Not done			
pn525	53.8	38.5	7.7	52	42.0	58.0	0	88
pn680	100	0	0	19	94.5	5.5	0	91
pn720	98.4	1.6	0	64	96.3	3.7	0	81
pn770	96.3	3.7	0	109	26.8	38.0	35.2	106
pSH	95.1	4.9	0	616	28.3	60.6	11.1	198

FIG. 4. Intracellular localization of wild-type and mutant forms of ICP0 in the presence or absence of ICP27 in transfected cells. Vero cells were cotransfected with pSH or a mutant ICP0 plasmid (pn428, pn525, pn680, pn720, or pn770) with or without the plasmid expressing ICP27 (pKHX-BH). At 24 h posttransfection, monolayers were processed for IF. Rabbit anti-ICP0 polyclonal antibody (J17) was used as the primary antibody. IF-positive cells were categorized and counted as described in the legend to Fig. 2. The percentage of IF-positive cells that fell into each category is shown in the table on the right; a diagram indicating the forms of ICP0 expressed by the mutant plasmids is shown on the left. The locations of the IE genes encoding ICP0, ICP4, ICP22, ICP27, and ICP47 are shown by arrows on the HSV-1 genome at the top of the figure. Beneath the expanded map of ICP0 is a diagram of ICP0 mRNA, with the introns spliced out and coding sequences shown as black boxes. The three-digit number in the designation of pn mutant corresponds to the amino acid residue disrupted by the nonsense linker insertion (6).

derived N- and C-terminal ICP27 deletion and insertion mutant plasmids kindly provided by Rozanne Sandri-Goldin (30). For this purpose, Vero cells were transfected with 1 μg of pSH alone or were cotransfected with 1 μg of pSH and 1 μg of either the plasmid expressing wild-type ICP27 (pSG130B/S) or a mutant form of ICP27 (N9M, R3IF, S5, N6, F21, S13, S1B, and S18). In these tests, mutant forms of ICP27 with alterations in the N-terminal half of ICP27 (amino acids 1 to 261 [mutant plasmids N9M, R31F, S5, N6, and F21]) retained nearly wild-type ability to inhibit the nuclear localization of ICP0 (data not shown). Consistent with the results described above (Fig. 3), plasmids with mutations in the C-terminal half of ICP27 (amino acids 262 to 512 [mutant plasmids S13, S1B, and S18]) exhibited significantly reduced ability to inhibit the nuclear localization of ICP0 (data not shown). Taken together, the results of mapping studies indicate that the C-terminal half of ICP27 is absolutely required for its inhibitory effect on the nuclear localization of ICP0 and that small mutations in the N-terminal half do not significantly alter this effect.

The region of the ICP0 molecule between amino acid residues 720 and 769 is required for responsiveness to the negative effect of ICP27. (i) Transfections. To identify the region of the ICP0 molecule that responds to ICP27, Vero cells were cotransfected with 1 μg of pSH or 1 μg of the ICP0 nonsense mutant plasmids pn428, pn525, pn680, pn720, and pn770, with or without plasmid pKHX-BH, which expresses the wild-type form of ICP27 (Fig. 4).

These tests demonstrate that in the absence of ICP27, the wild-type (pSH) and mutant forms of ICP0 specified by pn680, pn720, and pn770 localized almost exclusively to the nuclei of transfected cells. The ICP0 peptide specified by pn525 localized predominantly, but not exclusively, to the nucleus. This altered localization pattern may be due to the fact that the nonsense linker in this plasmid is located very near the highly basic amino acid sequence (amino acids 501 to 506) that is thought to constitute a portion of the nuclear localization signal of ICP0 (21). The ICP0 peptide specified by pn428, which lacks the nuclear localization signal and is sufficiently small to diffuse throughout the cell (62), was localized nearly equally within both the cytoplasm and nuclei of IF-positive cells.

In the presence of the wild-type ICP27-expressing plasmid, pKHX-BH, nuclear localization of wild-type ICP0 and the mutant form of ICP0 specified by pn770 was strongly inhibited. In contrast, nonsense mutations in pn720 and pn680, which specify proteins lacking progressively greater amounts of the C-terminal portion of ICP0, were not inhibited. These observations demonstrate that amino acid residues 720 to 769 play a major role in the ability of ICP27 to inhibit the nuclear localization of ICP0. They also suggest that the region of ICP0 that responds to ICP27 is distinct from the nuclear localization signal of ICP0.

(ii) Infections. To determine whether the patterns of ICP0 localization observed in transfections were reproducible in viral infections, ICP0 mutant viruses (n525, n680, n720, and n770) which contain the same mutations as plasmids pn525, pn680, pn720, and pn770, respectively, were used to infect Vero cells. Figure 5 shows that in n770- and KOS-infected cells, ICP0 was localized predominantly but not exclusively to nuclei. However, in n680- and n720-infected cells, ICP0 was localized exclusively to nuclei. In n525-infected cells, ICP0 was present in both nuclei and cytoplasm, the stronger signal occurring in the nucleus.

It should be noted that the intranuclear staining patterns of the ICP0 peptides specified by n720, n680, and n525 were quite different from those of KOS and n770, in that staining was more diffuse in the former and more punctate in the latter. Also, ICP0-specific nucleolar staining was never observed. These findings are consistent with the observations of Everett (21). As expected, the ICP0 intracellular staining pattern was nearly identical in n720- and 5dl1.2-infected cells, i.e., the pattern of ICP0-specific fluorescence produced by a mutant virus lacking the responsive region of the ICP0 molecule (n720) was indistinguishable from that of a mutant virus lacking ICP27 (5dl1.2). The localization of ICP4 was the same in all ICP0 mutant- and KOS-infected cells (data not shown).

As suggested by the results of transfection assays and confirmed by observations made for virus-infected cells, the region of the ICP0 molecule between amino acid residues 720 and 769 is required for responsiveness to the negative effect of ICP27 on the nuclear localization of ICP0.

Correlation between the level of ICP27 expression and its inhibitory effect on the nuclear localization of ICP0. Several lines of evidence support the concept that the level of ICP27 expression determines the extent of its inhibitory effect on the nuclear localization of ICP0. First, as shown in Fig. 2, the more ICP27-expressing plasmid, pKHX-BH, that is included in cotransfections with pSH, the greater is the inhibitory effect on the nuclear localization of ICP0. Second, in cells infected with 5dl1.2 (i.e., by a virus lacking ICP27), ICP0 was localized exclusively to nuclei (Fig. 1C and 5). Third, in n12-infected cells, which express no functional ICP4 but high levels of both ICP0 and ICP27 (due to the inability of the n12 form of ICP4 to down-regulate IE gene expression), ICP0 was localized predominantly within the cytoplasm (Fig. 1B).

However, it is difficult to study the concentration effect of ICP27 on the nuclear localization of ICP0 in infected cells, because the expression of ICP0, ICP4, and ICP27 is tightly regulated. Furthermore, the positive effect of ICP4 and the negative effect of ICP27 on the nuclear localization of ICP0

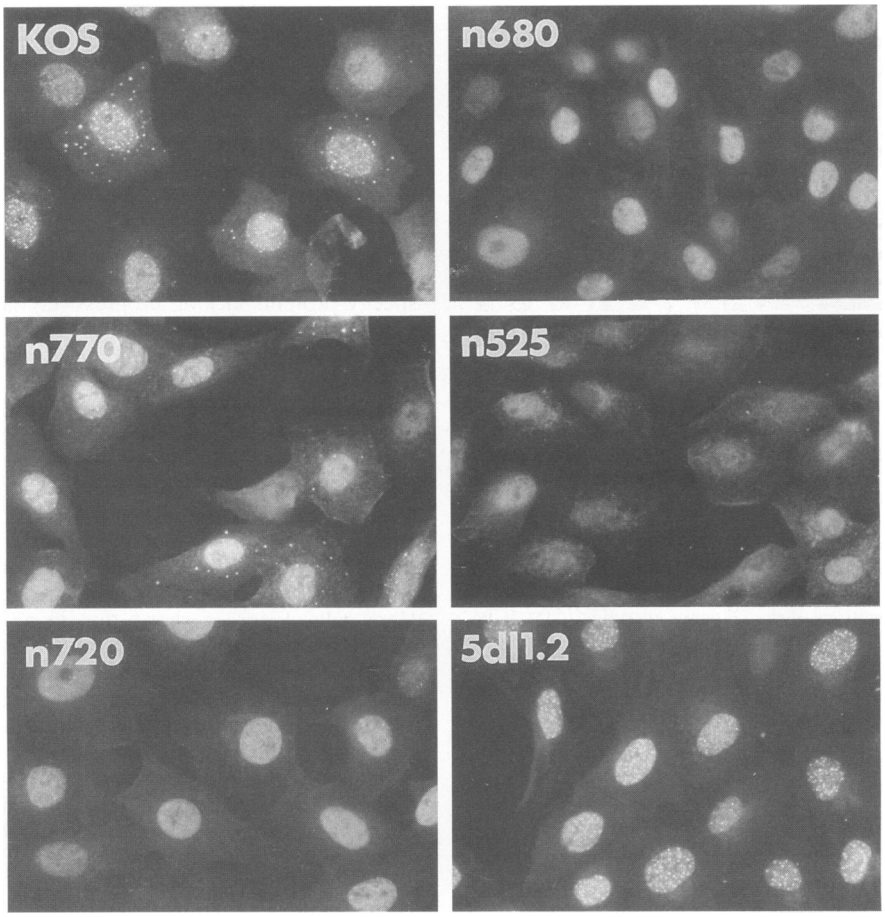


FIG. 5. Intracellular localization of mutant and wild-type ICP0 peptides in virus-infected cells. Vero cells were infected with 20 PFU of KOS or ICP0 mutant viruses (n525, n680, n720, and n770) per cell or with the ICP27 deletion mutant virus, 5dl1.2, as indicated. At 5 h postinfection, monolayers on coverslips were processed for IF. MAb H1083, specific for ICP0, was used as the primary antibody, as described in the legend to Fig. 1.

occur simultaneously in infected cells. To overcome this difficulty, we chose to use cells infected with the ICP4 null mutant, n12, to study the effect of ICP27 concentration during virus infection. The rationale for using n12-infected cells was that only two proteins (ICP0 and ICP27) are expressed and that the positive effect of ICP4 is absent. If the concentration of ICP27 is indeed a critical factor, the pattern of ICP0 localization in cells infected with n12-infected cells should also be multiplicity dependent (i.e., dependent on the level of expression of ICP27). Thus, in these tests, ICP0 should be localized predominantly to nuclei in cells infected with n12 at low multiplicities and to nuclei and cytoplasm at higher multiplicities. To test the effects of ICP27 concentration during infection, Vero cells were infected with n12 at multiplicities of 0.5, 5, and 20 PFU per cell, and the localization of ICP0 was examined by IF. The ICP27 mutant virus, 5dl1.2, and KOS were included as controls at each multiplicity tested. The results of these tests revealed the following (Fig. 6). (i) In cells infected with n12, the lower the multiplicity of infection was, the greater was the number of ICP0-positive cells that exhibited exclusively nuclear staining. Thus, at a multiplicity of 0.5 PFU per cell, about 50% of ICP0-positive cells exhibited exclusively nuclear staining (Fig. 6A). In contrast, the higher the multiplicity of n12 was, the greater was the number of cells exhibiting cytoplasmic staining (compare Fig. 6A, B, and C). (ii) At all multiplicities tested, ICP0 was always localized exclusively to the nuclei of 5dl1.2-infected cells (Fig. 6D, E, and F). (iii) At all multiplicities of KOS tested, the intracellular localization of ICP0 was unchanged in that it was always localized prodominantly to nuclei, a lesser proportion being present in the cytoplasm, as shown in Fig. 1 and 5 (data not shown). Moreover, the localization pattern of ICP27 in n12-infected cells also changed as a function of multiplicity. As multiplicities of infection increased, an increasing number of cells exhibited ICP0- and ICP27-specific cytoplasmic staining. Compare Fig. 1B and L with Fig. 7a, panels A and B.

Although these studies suggest that the concentration of ICP27 is critical in determining the intracellular staining pattern of ICP0, a potential problem exists with the use of n12-infected cells. As mentioned above, the levels of ICP0 and ICP27 increased in the absence of functional ICP4, and they also increased as the multiplicity increased. Thus, negative effects on the nuclear localization of ICP0 can result both from the increasing concentration of ICP27 and from the increasing concentration of ICP0 itself in this system. In transient assays, however—and independently of the amount of ICP0-expressing plasmid used in these transfections—cells expressing ICP0 exclusively in the cytoplasm or in the cytoplasm to a greater extent than in the nucleus were never observed (Fig. 2b). Therefore, despite the negative effect that may result from the

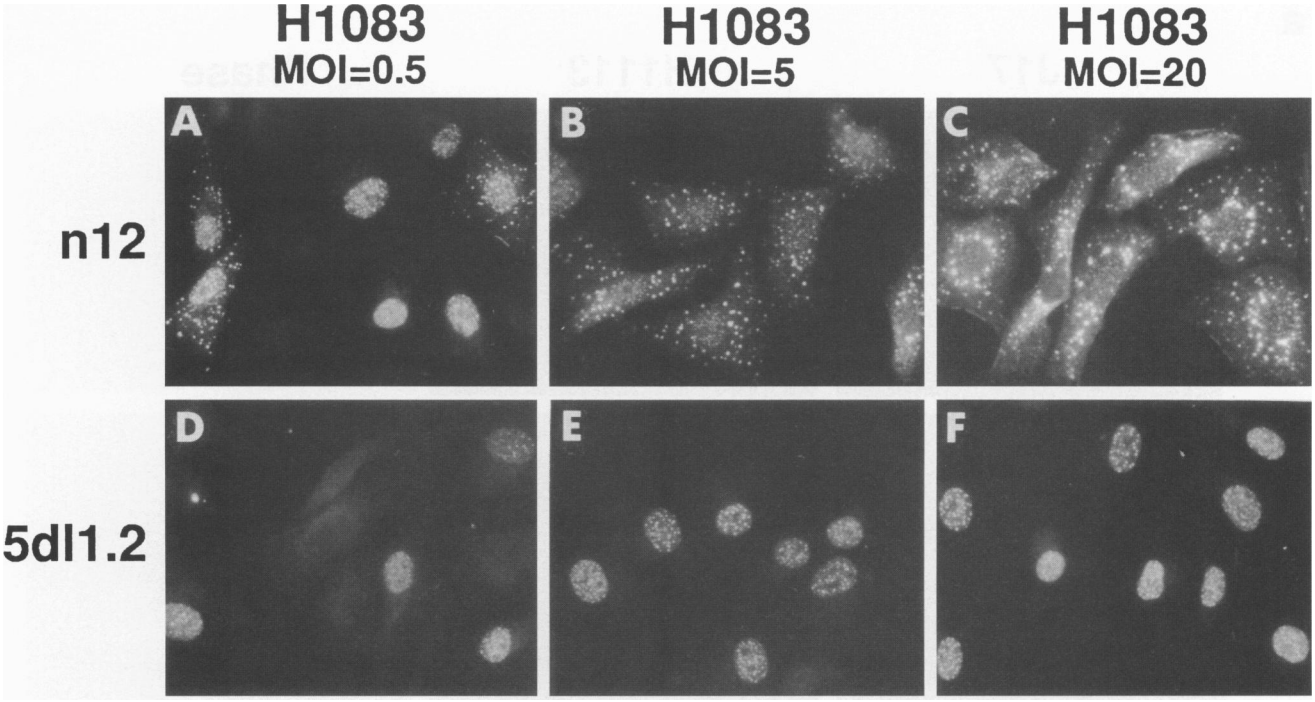


FIG. 6. Localizations of ICP0 in cells infected with 0.5, 5, or 20 PFU of n12 or 5dl1.2 per cell. Vero cells were infected with n12 or 5dl1.2 at the multiplicities indicated and were processed at 5 h postinfection for IF with anti-ICP0 MAb H1083, as described in the legend to Fig. 1.

increased level of expression of ICP0 as a result of increased multiplicity, the negative effect of ICP27 is critical for the predominantly cytoplasmic staining pattern of ICP0 at high multiplicity, and this negative effect increases as the multiplicity increases.

As a second approach to correlating the localization pattern of ICP0 with the expression level and localization pattern of ICP27, a double staining experiment was performed (Fig. 7). In contrast to the experiments described above, this approach permits one to examine the relative levels of expression and the staining patterns of ICP0 and ICP27 simultaneously within the same cell. For this purpose, Vero cells were infected with n12 or KOS at 0.5 PFU per cell. At 5 h postinfection, the cells were reacted with a combination of an ICP0-specific rabbit polyclonal antibody (J17) and an ICP27-specific mouse monoclonal antibody (H1113) as primary antibodies and then incubated with FITC-conjugated goat anti-rabbit antibody and RITC-conjugated goat anti-mouse antibody (Fig. 7a). In n12-infected cells that expressed relatively high levels of ICP27 in both the nucleus and cytoplasm, ICP0 was present either exclusively in the cytoplasm or in the cytoplasm to a greater extent than in the nucleus (cells with white arrows), whereas in cells in which ICP27 staining was predominantly nuclear, ICP0 was also found predominantly in the nucleus (cells with open arrows). In contrast, in KOS-infected cells, the intracellular localizations of ICP0 and ICP27 were similar in all IF-positive cells.

As controls for the double staining procedure, Fig. 7b demonstrates that the rhodamine and fluorescein stains did not interfere with each other, as shown by the unique staining patterns obtained with the two chromophores.

Notably, although double staining permits one to examine ICP0 and ICP27 within the same cells, it cannot ascertain whether the alteration in the localization of ICP0 in n12-infected cells is due to ICP27 or to a parallel effect resulting from the lack of ICP4. In an attempt to address this question, an additional double staining experiment in which cells were cotransfected with plasmids expressing ICP0 and ICP27 was performed. This experiment permits one to examine the levels and the patterns of localization of ICP0 and ICP27 when no other viral proteins are present. In this experiment, Vero cells were cotransfected with 1 μg each of the plasmids expressing wild-type ICP0 and ICP27. Twenty-four hours after transfection, monolayers were processed for IF. Cells were double stained for ICP0 and ICP27 as described in the legend to Fig. 7. In interpreting the results of these tests, it should be noted that in cells transfected with the ICP27-expressing plasmid alone, only a very few IF-positive cells were detected and the intensity of staining was quite low (data not shown). In cotransfection experiments, the intensity of ICP27 staining when present only in the nucleus was also weak but was considerably stronger in cells expressing ICP27 in both the nucleus and cytoplasm.

The following general observations were made from these cotransfection experiments (Fig. 8). In more than half of the ICP0-positive cells examined, ICP0 was detected exclusively in the nucleus (category N). In two-thirds of these cells, ICP27 was not detectable, and in one-third it was detected only weakly in the nucleus, implying that the absence or low level of expression of ICP27 favors the nuclear localization of ICP0. In one-third of the ICP0-positive cells examined, ICP0 was detected either predominantly in the nucleus or equally in the nucleus and cytoplasm (category N≥C). In these cells, ICP27 was detected in nearly all cells, a significant cytoplasmic component being present in two-thirds of them and an exclusively nuclear component being present in only one-third. Only 16% of the ICP0-positive cells exhibited predominantly or exclusively cytoplasmic staining (category N<C or C). A significant cytoplasmic component of ICP27 staining was present in 100% of these cells. These findings indicate that the

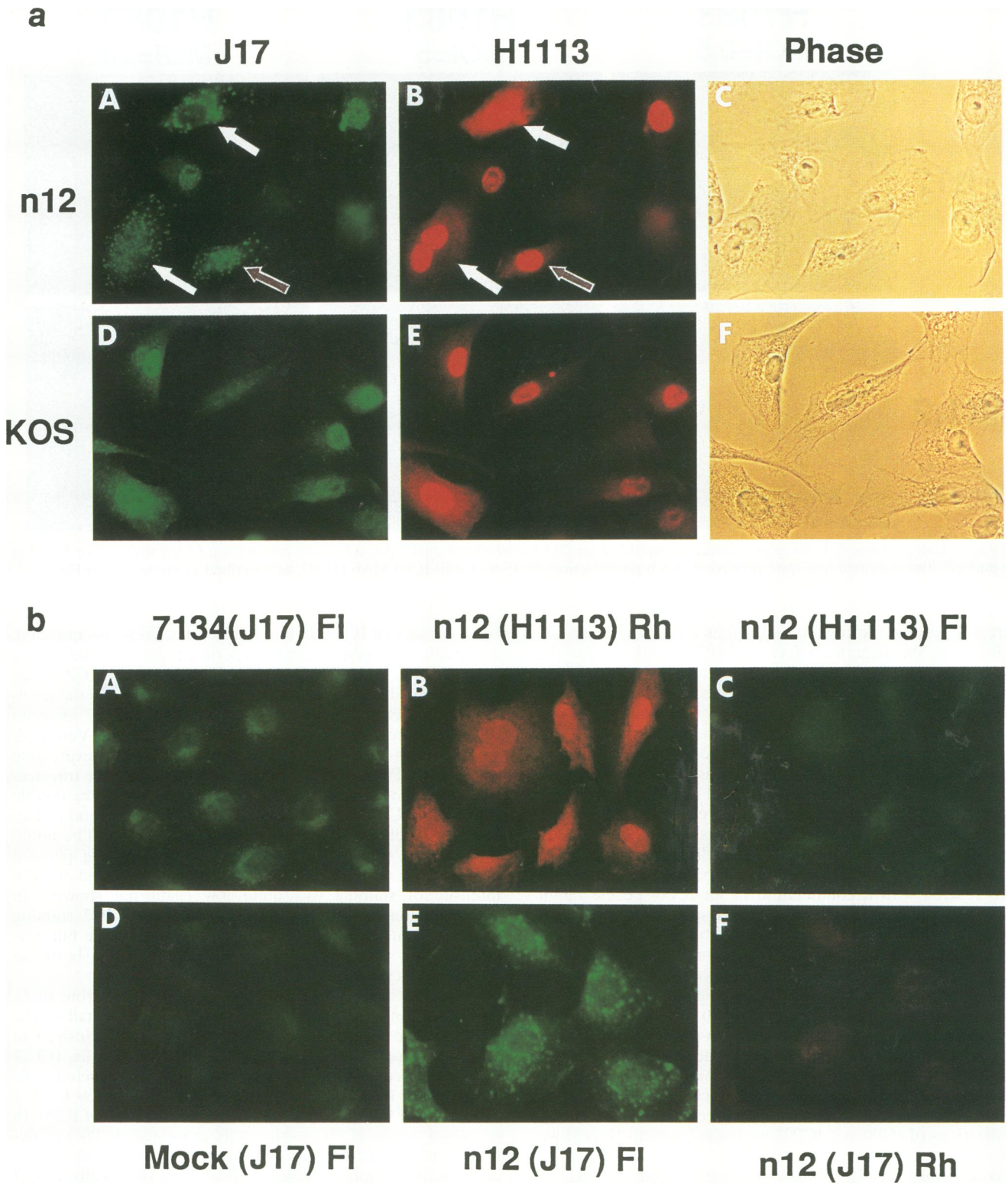


FIG. 7. Comparison of the localizations of ICP0 and ICP27. (a) Vero cells were infected with either n12 or KOS at 0.5 PFU per cell and were processed for IF at 5 h postinfection, as described in the legend to Fig. 1, except that the cells were double stained with J17 (rabbit anti-ICP0 polyclonal antibody) and H1113 (mouse anti-ICP27 MAb) as primary antibodies. RITC-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit immunoglobulin antibodies were used as secondary antibodies. ICP0-specific staining is shown in green, and ICP27-specific staining is shown in red. Phase-contrast micrographs are shown on the right side. (b) Vero cells were infected with 7134 or n12 at 20 PFU per cell or were mock infected. At 5 h postinfection, monolayers were processed for IF as described in the legend to Fig. 1, except that panels A and D, which show 7134-infected and mock-infected cells, respectively, were stained with J17 and were photographed with the Fl filter; panels B and C, which show

cytoplasmic localization of ICP27 correlates with the cytoplasmic localization of ICP0.

The results presented in Fig. 8 provide more convincing evidence that the alterations in the localization of ICP0 and ICP27 in n12-infected cells are not due to parallel effects resulting from the lack of ICP4; rather, the concentration and localization pattern of ICP27 are key factors in determining the intracellular localization pattern of ICP0.

Collectively, the data presented in Fig. 6, 7, and 8 indicate that the level of ICP27 expression and the localization pattern of ICP27 are critical for its inhibitory effect on the nuclear localization of ICP0 in transfected and infected cells.

PROTEIN	CATEGORY								
ICP0	N			N≥C			N<C or C		
	222 (53)			135 (31)			67 (16)		
ICP27	O	N	N≥C	O	N	N≥C	O	N	N≥C
	155(70)	66(30)	1(0)	4(3)	37(27)	94(70)	0(0)	0(0)	67(100)

FIG. 8. Localization of ICP0 and ICP27 in cells cotransfected with plasmids expressing wild-type ICP0 and ICP27. The staining patterns of ICP0 and ICP27 in cotransfected cells are indicated as follows: N, staining detected only in the nucleus; N≥C, nuclear staining greater than or equal to cytoplasmic staining; N<C, nuclear staining less than cytoplasmic staining; C, staining detected only in the cytoplasm; O, no staining detected in either nucleus or cytoplasm. For the ICP0 row, data are numbers of ICP0-positive cells in each category; the percentages of total ICP0-positive cells are given in parentheses. For the ICP27 row, data are numbers (percent) of ICP0-positive cells in each category not expressing ICP27 (O), expressing ICP27 exclusively in the nucleus (N), or expressing ICP27 in the nucleus to an extent greater than or equal to that in the cytoplasm (N ≥ C).

DISCUSSION

Studies conducted in mammalian (3, 25), *Drosophila* (55, 56, 64), and yeast (40, 43) systems have demonstrated a close correlation between the activities of cellular transcriptional regulatory proteins and the localization patterns of these proteins within cells. Protein phosphorylation states and protein-protein interactions have both been shown to play definitive roles in regulating the intracellular localization patterns and activities of proteins (for a review, see references 27, 32, and 62). With the exception of experiments demonstrating a correlation between the phosphorylation state and intracellular localization of simian virus 40 T antigen (33, 53, 54), however, few studies have been conducted in viral systems. The findings presented in this paper not only shed new light on the complex mechanism of HSV gene regulation but also demonstrate that HSV is a useful model system in which to examine the relationship between the activities of viral regulatory proteins and their intracellular locations.

ICP27 and ICP4 affect the intracellular localization of ICP0. Following infection with wild-type HSV-1, ICP0 is localized predominantly, but not exclusively, to the cell nucleus. In these studies, we have shown that ICP27 inhibits and that ICP4 promotes the nuclear localization of ICP0 in both mutant virus-infected cells and in two-plasmid transient expression assays.

While these studies clearly demonstrate that ICP27 and ICP4 affect the localization pattern of ICP0 in both ICP27 and ICP4 null mutant virus-infected cells, it will be difficult to determine whether these effects are a consequence of the use of null mutant viruses or whether they actually occur during HSV-1 replication. The demonstration by others that the levels and kinetics of synthesis of ICP0, ICP27, and ICP4 mRNAs (18, 68) differ relative to one other throughout the wild-type replication cycle and our demonstration that the effect of ICP27 on the localization of ICP0 is concentration dependent would support the hypothesis that these effects occur during wild-type virus replication. If they do, they would have significant implications for the mechanism underlying the regulation of HSV gene expression.

Thus, for example, the positive effect of ICP4 on the nuclear localization of ICP0 may serve to promote the synergistic transactivating activity of ICP0 and ICP4 (19, 24, 44, 49). One might postulate that only when ICP0 is present in the nucleus simultaneously with ICP4 can the synergistic enhancing activity of ICP0 be expressed. This scenario is consistent with the recent demonstration that ICP0 and ICP4 interact directly with each another through carboxy-terminal sequences (73) and with elements of the cellular transcription machinery (29, 63, 74). Regardless of whether ICP27 and ICP4 affect the intracellular localization of ICP0 in wild-type virus-infected cells, the observation that ICP0 is present in the cytoplasm of cells infected with ICP4-deficient mutants provides an explanation for the long-recognized paradox that the abundant ICP0 present in ICP4 null mutant-infected cells is unable to activate E gene expression (16).

Although ICP27 and ICP4 are able to affect the *intracellular* localization of ICP0 in transfected and infected cells, neither protein had a major effect on the *intranuclear* staining pattern of ICP0 in these cells. This is in contrast to the report of Gelman and Silverstein that ICP4 is required for the uniform distribution of ICP0 in the nucleus (24). The basis for this discrepancy is unclear; however, the two studies differed in the cell type used and in several procedural details.

The potential role of phosphorylation in determining the intracellular localization and regulatory activities of IE proteins. A major consideration in attempting to establish the significance of the observed effects of ICP27 and ICP4 on the intracellular localization of ICP0 is the molecular mechanism by which these proteins might achieve their effects. All three proteins are nuclear phosphoproteins, and phosphate has been shown to cycle on and off of each of them during the course of viral infection (1, 46, 69). Moreover, multiple phosphorylated forms of all three proteins have been detected by two-dimensional isoelectrofocusing (1).

Of the three proteins, most is known about the role of phosphorylation in modulating the activities of ICP4. ICP4 migrates as three differentially phosphorylated species (4a, 4b, and 4c) on SDS-polyacrylamide gels (1, 17, 41, 46, 69). The fastest migrating species, 4a, can be chased into the more slowly migrating species, 4b and 4c. Species 4a accumulates in the cytoplasm, whereas the two more slowly migrating species

the same field of n12-infected cells, were stained with H1113 and were photographed with the Rh and the Fl filters, respectively; panels E and F, which show the same field of n12-infected cells, were stained with J17 and were photographed with the Fl and Rh filters, respectively. The Rh and Fl filters allow one to view rhodamine and fluorescein staining, respectively. Part b serves as a control for part a. The leftmost two panels in part b show the ICP0 specificity of the J17 antibody; the middle and right-hand panels show that no interference occurred between the two chromophores that were used.

localize to the nucleus (41, 46). Only nuclearly localized ICP4 is able to activate transcription (16). Of special significance to the present study is the ability of ICP27 to alter the electrophoretic mobility of ICP4, such that the ICP4 species synthesized in cells infected with ICP27 null mutants migrate more slowly in SDS-polyacrylamide gels than species synthesized in cells infected with wild-type virus (39, 50). Similarly, in extracts of cells cotransfected with plasmids expressing ICP4 and ICP27, only the faster migrating species are detected, whereas in extracts of cells transfected with the ICP4-expressing plasmid alone, only the more slowly migrating species are detected (66). It is not clear whether the faster and more slowly migrating species of ICP4 described in these studies correspond to 4a and to 4b and 4c, respectively. The ability of ICP27 to affect the electrophoretic mobility of ICP4 is thought to be due to differences in phosphorylation (70), leading investigators to postulate that ICP27 may possess kinase or phosphatase activity. Although this is an attractive hypothesis, no such activity has been demonstrated for ICP27. An alternative hypothesis is that ICP27 affects the activities of viral and/or cellular kinases or phosphatases which, in turn, modify IE proteins.

Also relevant to the potential role of phosphorylation in determining the functional properties of ICP4 are recent studies by Xia and DeLuca which demonstrate a correlation between impaired viral growth and reduced phosphorylation of ICP4 (71).

Potential roles for phosphorylation in regulating the transcriptional activities of ICP0 and ICP27 have also been postulated. Biochemical and functional characterization of the species of ICP0 and ICP27 present in the nucleus and in the cytoplasm will clearly be necessary to determine whether the phosphorylation states of these proteins regulate their intracellular localizations.

The C-terminal half of ICP27 is required for its negative effect on the nuclear localization of ICP0. Mutational analysis of ICP27 has shown that the intact C-terminal half of ICP27 is required for its negative effect on the nuclear localization of ICP0. This region of ICP27 is also required for its *trans*-activating and *trans*-repressing functions (30, 39, 51). It is likely that the amino-terminal half of ICP27 will also prove to be important for ICP27's inhibitory effect on the nuclear localization of ICP0, since the nuclear localization signal is located in the amino-terminal half of the protein (30) and an acidic amino-terminal region of ICP27 has recently been shown to be required for an essential lytic function (52). Evidence of a role for the N-terminal half of ICP27 in inhibiting the nuclear localization of ICP0 awaits the construction and testing of suitable mutant viruses.

The inhibitory effect of ICP27 on nuclear localization is likely specific for ICP0. In view of the inhibitory effect of ICP27 on the nuclear localization of ICP0, it is intriguing to ask whether ICP27 has similar effects on other HSV nuclear proteins. In an effort to address this question, we asked whether ICP27 can inhibit the nuclear localization of ICP8, the major single-stranded DNA-binding protein of HSV-1. Although recent work by Curtin and Knipe suggests that ICP27 may promote the proper folding and/or intranuclear localization of ICP8 (12), these authors presented no evidence for a direct interaction between the two proteins or for the modification of one protein by the other. Work conducted in our laboratory has shown that ICP27 has no effect on the intracellular localization of ICP8 (unpublished observations). However, when the ICP0 amino acid sequence that responds to ICP27 was inserted in frame into the ICP8 protein, a partial but reproducible inhibitory effect of ICP27 on the nuclear localization of the ICP8-ICP0 fusion protein was observed (unpublished observations). These findings suggest that ICP27 does not have a general effect on the intracellular localization of all nuclear proteins but that its effect on ICP0 is specific.

The C-terminal region of ICP0 is responsive to the effect of ICP27. The region of ICP0 responsive to the negative effect of ICP27 was mapped in this study to the C terminus of the protein, between amino acid residues 720 and 769. Several other functions of ICP0 have also been mapped to this region, including (i) the major transactivation domain of ICP0 (6), (ii) the domain responsible for the synergistic activation of HSV gene expression by ICP0 and ICP4 (21), (iii) the ability of ICP0 to interact physically with ICP4 and with TBP (73), and (iv) the ability of ICP0 to associate with virions during assembly (72). In view of the multiplicity of functions associated with this portion of the ICP0 molecule, it is not surprising that mutations in this region produce unusual staining patterns (reference 21 and the present study). By extension, it is likely that the alterations in staining patterns observed reflect the functional phenotypes of these mutant forms of ICP0.

The positive effect of ICP4 on the nuclear localization of ICP0. Previous studies by Knipe and Smith (35) have shown that in ICP4 *ts* mutant (*ts*756)-infected cells, ICP0 was present predominantly in the cytoplasm at the nonpermissive temperature. These authors suggested that the mutant form of ICP4 specified by *ts*756 interacts with ICP0, serving to retain ICP0 in the cytoplasm. Although this explanation may be correct for the *ts*756 form of ICP4, our studies of a large number of ICP4-deficient mutants suggest an alternative explanation. In these studies, ICP0 was present predominantly in the cytoplasm of cells infected with a variety of ICP4 mutant viruses including nonsense, deletion, and *ts* mutants (including *ts*756) (unpublished results). Because some of the ICP4 mutant peptides constitute only one-fourth to one-fifth of the intact molecule, it is unlikely that all of these mutant forms of ICP4 interact with ICP0 to result in its retention in the cytoplasm. It is more likely that the absence of functional ICP4 in ICP4$^-$ mutant-infected cells results in the overexpression of ICP27, which is ultimately responsible for the predominantly cytoplasmic localization of ICP0. In a similar vein, in wild-type virus-infected cells, ICP4 has a positive effect on the nuclear localization of ICP0 as well as the ability to repress the expression of ICP27. Therefore, in cells infected with wild-type virus, a portion of ICP4's positive effect on ICP0 may be indirect by inhibiting ICP27's negative effect.

The studies presented in this paper document cooperativity among three HSV-1 IE proteins in determining their intracellular locations. The increasing evidence that ICP22, a fourth IE nuclear phosphoprotein, also plays a regulatory role in HSV-1 gene expression (2, 48) may add an additional level of complexity to the already complex relationships that exist among these proteins. Additional functional and biochemical analysis of wild-type and mutant forms of ICP0, ICP4, ICP22, and ICP27 will be necessary to understand the molecular basis for and functional implications of these relationships.

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