Intracellular Localization of the Herpes Simplex Virus Type 1 Major Transcriptional Regulatory Protein, ICP4, Is Affected by ICP27

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Infected-cell protein 4 (ICP4) is the major transcriptional activator of herpes simplex virus (HSV) gene expression during productive infection. ICP0 has broad transactivating activity for all classes of HSV genes as well as cellular genes and genes of heterologous viruses. Together, the transactivating activities of ICP4 and ICP0 are synergistic. ICP27, which alone does not exhibit major transregulatory activity, is able to differentially activate and repress viral gene expression induced by ICP4 and ICP0. Thus, ICP27 plays a modulatory role in viral gene expression. In order to explore the functional relationships among ICP4, ICP0, and ICP27 in the regulation of viral gene expression, we have used indirect immunofluorescence to examine the intracellular localization of ICP4 in cells infected with wild-type virus or with mutant viruses that did not express functional forms of ICP0 or ICP27. Although ICP4 localized to both the nuclei and cytoplasm of cells infected with either the wild-type virus or an ICP0 null mutant virus, this protein was present exclusively in the nuclei of cells infected with an ICP27 null mutant virus, suggesting that ICP27 is able to inhibit the nuclear localization of ICP4 during virus infection. Transient expression assays with pairs of plasmids that express wild-type forms of ICP4 and ICP0 or of ICP4 and ICP27 demonstrated that ICP27 has a significant inhibitory effect on the nuclear localization of ICP4, confirming the observations made with the mutant-virus-infected cells. By using a plasmid expressing wild-type ICP4 and a series of ICP27 mutant plasmids in transient expression assays, the C-terminal half of ICP27 was shown to be required for its inhibitory effect on the nuclear localization of ICP4. In similar studies using a series of ICP4 mutant plasmids, the region of ICP4 responsive to wild-type ICP27 was mapped to the C-terminal portion of the molecule between amino acid residues 820 and 1029. The level of expression of ICP27 was shown to have a significant effect on the intracellular localization of ICP4 in transient assays. These findings are consistent with previous studies in which ICP27 was shown to have an inhibitory effect on the nuclear localization of ICP0 (Z. Zhu, W. Cai, and P. A. Schaffer *J. Virol.* 68:3027–3040, 1994). Thus, ICP27 has a significant inhibitory effect on the ability of the two major HSV type 1 (HSV-1) regulatory proteins to localize to the nucleus. Collectively, these findings indicate that cooperative regulation of HSV-1 gene expression may well involve intracellular compartmental constraints.

The 152-kb genome of herpes simplex virus type 1 (HSV-1) encodes more than 70 genes (33–35, 45). During productive infection, these genes are expressed coordinately as three major kinetic classes: immediate-early (IE), early (E), and late (L) (26). Viral gene expression is regulated at both the transcriptional and posttranscriptional levels (8, 23, 27, 59) by at least four viral proteins including the virion-associated protein, VP16, and three of the five recognized IE proteins, ICP0, ICP4, and ICP27 (1, 6, 30). The fourth IE protein, ICP22, also appears to be involved in regulation as noted below, but its roles are only poorly understood.

Although the precise manner in which the HSV regulatory cascade is orchestrated by IE proteins is unclear, many studies suggest that ICP4, ICP0, and ICP27 act cooperatively to regulate HSV-1 gene expression (15, 16, 19, 20, 36, 41, 42, 48, 50, 55). Thus, ICP4 activates transcription of E and L genes and represses its own transcription as well as that of the other IE genes (10, 11, 14, 46). ICP0 is able to transactivate all classes of viral genes (IE, E, and L) as well as a number of cellular and heterologous viral genes (4, 5, 15–20, 38, 39, 41, 42, 48). ICP4 and ICP0 activate E and L gene expression synergistically (15, 16, 19, 20, 41, 42, 48). ICP27 by itself appears to

have little effect on the expression of HSV genes (16, 55); however, it is able to further enhance or repress the transactivating activities of ICP4 and ICP0 (16, 36, 50, 52, 55, 58). Recent studies suggest that ICP22 is also involved in the regulation of HSV gene expression (2, 54). This effect is apparently achieved by enhancing the level of ICP0 expression in a cell type-specific manner (47). The more recent finding that ICP22 is involved in modification of cellular RNA polymerase II further suggests that ICP22 plays a role in the regulation of HSV gene expression (48a, 51). The manner in which the activities of individual IE proteins are regulated and the mechanisms underlying the demonstrated cooperativity among ICP0, ICP4, and ICP27 are not understood.

Following synthesis in the cytoplasm, ICP4, ICP0, and ICP27 migrate to the nucleus where they perform their respective transregulatory functions. ICP4, the major viral transcriptional regulatory protein, exerts both its repressing and activating activities in the nucleus through a mechanism involving interaction with the general transcription factors, TBP and TFIIB (24, 57). We have demonstrated previously that the nuclear localization of ICP0 can be enhanced by ICP4 and inhibited by ICP27 (62). Here, we report that ICP27 also has a strong inhibitory effect on the nuclear localization of ICP4 in both infected and transfected cells. Moreover, the level of ICP27 expression and the localization pattern of ICP27 are critical for

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its inhibitory effect on ICP4. Although the mechanism by which ICP27 controls the intracellular localization of ICP0 and ICP4 is unclear, the ability of ICP27 to achieve this effect suggests the possibility that coordinate regulation of HSV gene expression involves intracellular compartmental constraints.

MATERIALS AND METHODS

Cells and viruses. All infections and transfections were performed in Vero cells. Vero cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HSV-1 strain KOS was used as the wild-type virus; all mutant viruses used in this study were derived from strain KOS. The ICP0 null mutant virus, 7134 (4), and the ICP22 mutant virus, 22/n199 (2), were propagated and assayed in Vero cells as described previously (53). The ICP22 mutant virus 22/n199, containing a nonsense linker at codon 200 of the ICP22 coding region, specifies a truncated peptide about half the size of the wild-type protein. The ICP27 deletion mutant virus, *5dl1.2* (32), and the ICP4 nonsense and deletion mutant viruses, *n214*, *n208*, *n18*, *n215*, *d156*, and *d2* (13), were propagated and assayed on 3-3 and E5 cells, respectively (12, 32). 3-3 and E5 cells are Vero cells stably transformed with the genes for ICP27 and ICP4, respectively.

Plasmids. The plasmids used in transient expression assays include the following. (i) Plasmid pn11 contains the wild-type ICP4 gene from strain KOS. ICP4 nonsense and deletion mutant plasmids, pn6, pn7, pn9, pn10, pASac, and pn3A28, kindly provided by N. DeLuca (University of Pittsburgh, Pittsburgh, Pa.) (12, 13), contain the same mutations as those in the KOS-derived mutant viruses, *n214*, *n208*, *n18*, *n215*, *d156*, and *d2*, respectively (13). (ii) Plasmid pKHX-BH contains the ICP27 gene from strain KOS and was kindly provided by S. Person (University of Pittsburgh). ICP27 nonsense (*n6*, *n7*, *n8*, *n9*, and *n11*) and deletion mutant plasmids (*d1*, *d2*, and *d3*) were derived from pKHX-BH (36). (iii) Plasmid pSG130B/S contains the ICP27 gene from strain KOS (M). ICP27 insertion and deletion mutant plasmids (pN9M, pR3IF, pS5, pN6, pF21, pS13, pS1B, and pS18) derived from pSG130B/S were kindly provided by R. M. Sandri-Goldin (University of California, Irvine) (25). (iv) Plasmid pSH contains the ICP0 gene from strain KOS (4).

Antibodies. Mouse monoclonal antibody H1113 is specific for ICP27 (1) and was kindly provided by L. Pereira (University of California, San Francisco). Monoclonal antibody 58S is specific for ICP4 and was generated from a hybridoma cell line obtained from the American Type Culture Collection (56). Rabbit polyclonal antibody R021, directed against ICP4 (9), was kindly provided by R. Courtney (Pennsylvania State University College of Medicine, Hershey). Rhodamine isothiocyanate (RITC)-conjugated goat anti-mouse and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (immunoglobulins G, A, and M) antibodies were obtained from Cappel Laboratories (Durham, N.C.).

Transfection. The calcium phosphate coprecipitation method was used in all transfections (7). Briefly, 24 h prior to transfection, 5×10^5 Vero cells were seeded in 60-mm-diameter petri dishes containing sterile 18-mm-diameter circular glass coverslips for use in immunofluorescence (IF) tests. Three hours prior to transfection, the medium was changed. For transfection, 10 μ g of total DNA (plasmid DNA plus salmon testis carrier DNA) was suspended in 250 μ l of 2 $\times$ BES transfection buffer [50 mM BES [N,N-bis(hydroxyethyl)-2-aminoethanesulfonic acid], 280 mM NaCl, 1.5 mM Na₂HPO₄; pH 6.95]. The DNA solution was mixed with an equal volume of 250 mM CaCl₂ and incubated at room temperature for 30 min, the suspension containing the DNA precipitate was added to the medium that overlaid cell monolayers, and transfected cultures were incubated at 37°C for 20 h, then washed with TBS (137 mM NaCl, 5 mM KCl, 25 mM Tris-HCl; pH 7.4), and refed with fresh medium and processed for IF 4 h later.

IF tests. Vero cells were grown on coverslips as described above. Twenty-four hours after seeding, cells were infected with wild-type or mutant viruses at a multiplicity of infection of 20 PFU per cell or less as indicated in the legends to Fig. 6 and 7 or were transfected by the calcium phosphate coprecipitation procedure with plasmid DNAs in the combinations and concentrations described in the legend to Fig. 2. Infected monolayers on coverslips were processed for IF at 5 h postinfection, and transfected monolayers were processed and fixed at 24 h posttransfection.

The indirect IF procedure used in this study was performed as follows (23). Cells on coverslips were fixed in 3.7% formaldehyde for 30 min, made permeable by immersion 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 (58S [1:50], R021 [1:150], and H1113 [1:600]) was added to monolayers on coverslips which were incubated for 30 min at 37°C in a humid chamber. Coverslips were then washed with PBS, and 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 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 some experiments (see Fig. 7 and Table 1), cells were doubly stained simultaneously for ICP4 and ICP27 with a mixture of R021 (rabbit

anti-ICP4 polyclonal antibody) and H1113 (mouse anti-ICP27 monoclonal antibody) as primary antibodies. RITC-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit immunoglobulin antibodies were used as secondary antibodies.

Monolayers were viewed with a Zeiss fluorescence microscope with a 40 $\times$, OIL, PH3, Plan-Neofluar objective. For detection of fluorescence from FITC- and RITC-conjugated antibodies and exclusion of interference between these two chromophores, appropriate filter sets were used as described previously (62). Photographs were taken with Kodak Tri-X Pan 400 film.

RESULTS

Intracellular localization of ICP4 in wild-type and mutant virus-infected cells. To determine whether ICP0, ICP22, and ICP27 are able to affect the intracellular localization of ICP4, we examined the fluorescence staining pattern of ICP4 in cells infected (20 PFU per cell) for 5 h with either wild-type virus or mutant viruses containing mutations in the genes encoding ICP0, ICP22, and ICP27 (Fig. 1).

The localization pattern of ICP4-specific fluorescence was similar in cells infected with KOS, the ICP22 nonsense mutant, 22/n199, and the ICP0 null mutant, 7134 (Fig. 1A, C, and F). In cells infected with these viruses, ICP4 was most prominent in globular replication compartments within the nuclei as previously described by Knipe et al. (28). Diffuse ICP4-specific fluorescence was also detected in both nuclei and cytoplasm. In contrast, the ICP4-specific staining pattern was very different in cells infected with the ICP27 deletion mutant, *5dl1.2* (Fig. 1B). In these cells ICP4 was detected exclusively in the nucleus, where it was diffuse and evenly distributed. Very few cells exhibited staining characteristic of replication compartments. Because DNA replication is deficient in *5dl1.2*-infected cells (only 18% of the wild-type level of DNA replication occurs in these cells; 32), the absence of replication compartments was not surprising. These observations suggest that the intracellular localization and particularly the intranuclear staining pattern of ICP4 characteristic of wild-type virus-infected cells are altered in the absence of ICP27 but not in the absence of ICP0 or ICP22.

The absence of both diffuse cytoplasmic and globular nuclear ICP4 staining in ICP27 deletion mutant virus-infected cells could be due to the reduction of DNA replication and/or L protein synthesis resulting from the absence of functional ICP27. To investigate the roles of these parameters in the localization pattern of ICP4, we examined the staining pattern of ICP4 in wild-type and mutant virus-infected cells in the presence of phosphonacetic acid (PAA), an inhibitor of HSV DNA synthesis (29, 31) (Fig. 1D and E), and in the presence of actinomycin D following removal of cycloheximide (Fig. 1G and H) (cycloheximide reversal). In contrast to the globular nuclear ICP4-specific staining pattern characteristic of cells infected with KOS, 22/n199, or 7134 in the absence of PAA (Fig. 1A, C, and F), ICP4 was detected as diffuse, even staining throughout the nuclei (excluding nucleoli) of cells infected with KOS (Fig. 1D) or 7134 or 22/n199 (not shown) in the presence of PAA. In addition, diffuse cytoplasmic staining was prominent in some of these cells (e.g., KOS [Fig. 1D]). As in the absence of PAA (Fig. 1B), ICP4 was also detected as diffuse, even staining throughout the nuclei (excluding nucleoli) of cells infected with the ICP27 deletion mutant *5dl1.2* in the presence of PAA (Fig. 1E). In neither case was diffuse cytoplasmic staining observed. These observations suggest that the absence of diffuse cytoplasmic ICP4-specific staining in *5dl1.2*-infected cells is not due to the absence of DNA replication but to the absence of ICP27.

Following cycloheximide reversal, only IE protein synthesis but not E-protein synthesis, viral DNA replication or L-protein synthesis, is permitted (26). Under this condition, diffuse

FIG. 1. Staining patterns of ICP4 in KOS- and mutant virus-infected cells. Vero cell monolayers on coverslips were either mock infected or infected (20 PFU per cell) with wild-type strain KOS or KOS-derived null mutant viruses carrying mutations in the gene for ICP27 (5dl1.2), ICP0 (7134), or ICP22 (22/n199). In experiments involving PAA, infected-cell monolayers were incubated in medium containing 400 μ g of PAA per ml and incubated at 37°C. At 5 h postinfection, monolayers were fixed, made permeable, and incubated with R021 rabbit anti-ICP4 polyclonal antibody as the primary antibody. FITC-conjugated goat anti-rabbit immunoglobulin antibodies were used as the secondary antibodies. In experiments involving cycloheximide (CH) reversal, Vero cell monolayers were preincubated for 1 h at 37°C in the presence of 75 μ g of CH per ml. They were then infected as described above with the indicated viruses in the presence of the same concentration of CH. At 5 h postinfection, CH-containing medium was removed, and the monolayers were washed and overlaid with medium containing no CH but 10 μ g of actinomycin D per ml. After a further 3 h of incubation, monolayers were processed for IF as described above. The viruses used to infect cells and the metabolic inhibitors used in selected experiments are indicated in the upper left-hand corner of each micrograph.

ICP4-specific staining was detected in the nuclei (excluding nucleoli) and cytoplasm of most cells infected with KOS (Fig. 1G) or 7134 or 22/n199 (data not shown), and in some cells, cytoplasmic staining was very strong. In contrast, diffuse ICP4-specific cytoplasmic staining was not detected in cells infected with 5dl1.2 (Fig. 1H). These observations suggest that the absence of diffuse ICP4-specific cytoplasmic staining in 5dl1.2-infected cells is due to the absence of ICP27 and not to the absence of L protein synthesis.

The results of these experiments indicate that the lack of diffuse ICP4-specific cytoplasmic staining in 5dl1.2-infected cells is due to the absence of functional ICP27. By extension, the presence of diffuse ICP4-specific cytoplasmic staining in cells infected with KOS, 7134, or 22/n199 must be due to the presence of functional ICP27 in these cells.

Effects of ICP0 and ICP27 on the intracellular localization of ICP4 in transient expression assays. In light of the observations described above, we next asked whether in transient assays, ICP27 or ICP0 was able to affect the localization of ICP4 in the absence of other HSV-specified gene products. In these experiments Vero cells were transfected with a plasmid expressing wild-type ICP4 (pn11) alone or together with plasmids expressing either wild-type ICP0 (pSH), ICP27 (pKHX-BH), or both at the indicated concentrations (Fig. 2). In these tests polyclonal rabbit anti-ICP4 serum, R021, was used as the primary antibody, although similar results were obtained with monoclonal antibody 58S. Cells exhibiting ICP4-specific fluorescence were counted and placed in one of five categories: cells that exhibited exclusively nuclear staining, cells that exhibited exclusively cytoplasmic staining, or cells that exhibited both nuclear and cytoplasmic staining, including cells in which nuclear staining was greater than, equal to, or less than cytoplasmic staining. Examples of cells included in each of these categories are shown in Fig. 2A. Figure 2B shows the total number of IF-positive cells examined and the percentage

of ICP4-positive cells that fell into each category following transfection with the indicated plasmid or plasmids. Although the percentages of IF-positive cells that fell into each category in each experiment varied somewhat, the overall trends in repeat experiments were the same. The fluctuation in the percentages seen from experiment to experiment probably reflects differences in difficult-to-control parameters such as the metabolic state of cells and transfection efficiency. It is notable that nuclear ICP4-specific staining characteristic of replication compartments was never detected in transfection experiments.

In cells transfected with a small amount (1 μ g) of the ICP4-expressing plasmid pn11, ICP4 appeared as diffuse staining, similar to that seen in virus-infected cells in the presence of PAA and was localized predominantly but not exclusively within the nuclei of IF-positive cells (Fig. 2B). In nearly all cells that exhibited both nuclear and cytoplasmic staining, ICP4-specific staining in the nucleus was much stronger than in the cytoplasm (e.g., Fig. 2A, N>C, second photo from the left). In cells transfected with larger amounts (5 or 10 μ g) of pn11 (experiment 3), the percentage of cells that exhibited exclusively nuclear staining was somewhat reduced compared with that of cells transfected with 1 μ g of pn11. In this experiment, the percentage of cells that exhibited both nuclear and cytoplasmic staining increased proportionally as the percentage of cells that exhibited staining only in the nucleus decreased. Cells expressing ICP4 exclusively in the cytoplasm or to a greater extent in the cytoplasm than the nucleus were never observed.

In cells cotransfected with small amounts (1 μ g of each) of pn11 (ICP4) and pSH (ICP0), the percentages of ICP4-positive cells in each category differed somewhat between experiments. In experiment 2 (but not in experiments 1 and 3, using 1 μ g of each plasmid), ICP0 had a moderate inhibitory effect on the nuclear localization of ICP4. This effect of ICP0 on the nuclear localization of ICP4 was also observed in experiment 3 in cells

FIG. 2. Localization of ICP4 in cells transfected with plasmids expressing ICP0, ICP4, and ICP27. Vero cells were transfected with pn11 (which expresses wild-type ICP4) alone or were cotransfected with pn11 and either pSH (which expresses wild-type ICP0) or pKHX-BH (which expresses wild-type ICP27) or with both pSH and pKHX-BH at the indicated concentrations. At 24 h posttransfection, monolayers were processed for indirect IF with the rabbit anti-ICP4 polyclonal antibody, R021. IF-positive cells were counted and categorized into one of five groups: cells that exhibited only nuclear staining (N), only cytoplasmic staining (C), nuclear staining greater than cytoplasmic staining (N>C), nuclear staining equal to cytoplasmic staining (N=C), or nuclear staining less than cytoplasmic staining (N<C). Examples of cells in each category are shown in panel A (photos of individual cells in N>C, N=C, and N<C are presented from left to right under N+C). The percentages of IF-positive cells that fell into each category and the total number (#) of IF-positive cells examined in each transfection are shown (B).

transfected with larger amounts of pn11 and pSH (5 μ g of each). Here, the percentage of ICP4-positive cells that exhibited exclusively nuclear staining was reduced relative to the percentage of ICP4-positive cells transfected with the same amount of pn11 alone (5 μ g). Notably, however, whether ICP0 and its inhibitory effect were present or not, ICP4 was always localized either exclusively or predominantly to the nuclei of nearly all IF-positive cells. Therefore, the inhibitory effect of ICP0 on the nuclear localization of ICP4 observed in some experiments parallels the concentration effect of ICP4 on its own localization, as observed in experiment 3 (Fig. 2B).

In contrast to ICP0, ICP27 had a strong inhibitory effect on the nuclear localization of ICP4 in all four transfection experiments conducted in the present study (see Fig. 2 to 4). Specifically, in cells cotransfected with pn11 and pKHX-BH, the percentage of cells that exhibited exclusively nuclear ICP4 staining was markedly reduced relative to that of cells transfected with pn11 alone, while the percentage of cells that exhibited both nuclear and cytoplasmic staining increased proportionally (Fig. 2B, experiments 1 and 2). Among ICP4-

positive cells that exhibited both nuclear and cytoplasmic staining, about half exhibited staining equally in the nucleus and in the cytoplasm.

In cells cotransfected with all three plasmids (Fig. 2B, experiments 1 and 2), the percentage of cells that exhibited exclusively nuclear staining was reduced even more dramatically, and a third category of cells, i.e., those that exhibited only cytoplasmic staining, was observed. These observations suggest that ICP27 in combination with ICP0 has a much stronger negative effect on the nuclear localization of ICP4 than does ICP27 alone. The mechanism involved in the enhancing effect of ICP0 on the inhibitory effect of ICP27 is unknown, but activation of ICP27 gene expression by ICP0 may be involved.

Collectively, the data from transfection studies indicate that in general, the ICP4-specific staining patterns observed in infected cells can be duplicated in large part in cells expressing only the IE proteins specified by transfected plasmids, indicating that other viral proteins are not required to establish these patterns. These data also demonstrate that ICP27 has an inhibitory effect on the nuclear localization of ICP4 in transient assays, which is enhanced in the presence of ICP0.

The C-terminal half of ICP27 is required for its negative effect on the nuclear localization of ICP4. To identify the region of the ICP27 molecule that mediates its inhibitory effect on the nuclear localization of ICP4, we cotransfected cells with pn11 and a series of ICP27 mutant plasmids. Vero cells were cotransfected with 1 μ g of pn11 and 1 μ g 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*) (36). All mutant plasmids used in these initial tests contained mutations within the C-terminal half of ICP27.

As shown in Fig. 3, cotransfection of pn11 with each of the ICP27 mutant plasmids produced a pattern of fluorescence similar to that seen in cells transfected with pn11 alone (Fig. 3, ICP4 alone). Notably, a mutant plasmid (*n11*), in which the C-terminal Phe is replaced by Leu, Ala, and Ser residues (36), also exhibited significantly reduced ability to inhibit the nuclear localization of ICP4 relative to that of wild-type ICP27 (pKHX-BH). On the basis of the mutational analysis shown in Fig. 3, no specific region within the C-terminal half of the ICP27 molecule was shown to be responsible for its inhibitory effect. Rather, either the entire C-terminal half of the ICP27 molecule or the conformation of the molecule is apparently required for its negative effect on the nuclear localization of ICP4.

In a further attempt to define the region of ICP27 required for its inhibitory effect, we tested a second series of KOS (M)-derived N- and C-terminal ICP27 deletion and insertion mutant plasmids kindly provided by Rozanne Sandri-Goldin (25). For this purpose, Vero cells were transfected with 1 μ g of pn11 alone or cotransfected with 1 μ g of pn11 and 1 μ g of either the plasmid expressing wild-type ICP27 (pSG130B/S) or a mutant form of ICP27 (N9M, R31F, 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 the wild-type ability to inhibit the nuclear localization of ICP4 (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 minimal ability to inhibit the nuclear localization of ICP4 (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

FIG. 3. Intracellular localization of ICP4 in cells transfected with plasmids expressing wild-type or mutant forms of ICP27. Vero cells were cotransfected with pn11 (which expresses wild-type ICP4) and either pKH-X-BH (which expresses wild-type ICP27) or plasmids expressing mutant forms of ICP27 (nonsense mutant plasmids n6, n7, n8, n9, and n11 and deletion mutant plasmids d1, d2, and d3). Twenty-four hours after transfection, monolayers were processed for IF. R021 polyclonal rabbit anti-ICP4 antibody was used as the primary antibody. IF-positive cells were categorized and counted as described in the legend to Fig. 2, except that categories N>C, N=C, and N<C are combined and presented as N+C. The percentages of IF-positive cells that fell into each category and the total number (#) of IF-positive cells examined in each transfection are shown on the right. 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 pKH-X-BH. The numbers at the ends of the polypeptides represent the amino acids which remain unaltered in the peptides. The names of the wild-type (WT) and mutant plasmids are shown on the left.

localization of ICP4 and that small mutations in the N-terminal half of ICP27 do not significantly alter this effect.

The region of the ICP4 molecule between amino acid residues 820 and 1029 is required for responsiveness to the negative effect of ICP27. (i) **Transfections.** To identify the region of the ICP4 molecule that responds to ICP27, Vero cells were either cotransfected with 1 µg of pn11, 1 µg of ICP4 nonsense mutant plasmids pn6, pn7, pn9, and pn10, or 1 µg of deletion mutant plasmids pΔSac and pn3Δ28, with or without plasmid pKH-X-BH which expresses wild-type ICP27 (Fig. 4).

These tests demonstrated that in the absence of ICP27, the wild-type and mutant forms of ICP4 specified by pn11, pn7, pn9, pn10, and pn3Δ28 were localized almost exclusively to the nuclei of transfected cells. The ICP4 peptides specified by pn6 and pΔSac were localized predominantly within the cytoplasm. These results confirmed (i) previous findings made with mutant viruses carrying the same mutations as those in pn6 and pΔSac and (ii) the fact that the nuclear localization signal (NLS) of ICP4 lies between amino acid residues 681 and 774 (13). Indeed, a stretch of positively charged amino acid residues homologous to the NLS of simian virus 40 T antigen is present in the sequence from amino acids 723 to 732 (13).

In the presence of ICP27, the nuclear localization of wild-type ICP4 (Fig. 4, bottom line) and, to a lesser extent, the mutant form of ICP4 specified by pn10 was significantly

inhibited (Fig. 4). In contrast, nonsense mutations in pn9 and pn7, which specify proteins lacking progressively greater amounts of the C-terminal portion of ICP4, were not inhibited and mutant pn3Δ28, containing an N-terminal deletion, was very weakly inhibited. These observations suggest that in addition to the C-terminal region of ICP4 (amino acids 820 to 1029), N-terminal residues may also be involved in the response of this protein to the negative effect of ICP27 or that the overall conformation of the ICP4 protein is important. These observations also suggest that the region of ICP4 that responds to ICP27 is distinct from the NLS of ICP4.

It should be noted that ICP27 was barely detectable in cells transfected with pKH-X-BH alone but was readily detectable and localized to both the nucleus and cytoplasm of the majority of ICP4-positive cells cotransfected with plasmids expressing wild-type ICP27 and wild-type ICP4. Similarly, ICP27 was barely detectable in ICP4-positive cells cotransfected with plasmids expressing wild-type ICP27 and mutant forms of ICP4. This result suggests that wild-type ICP4 can activate expression of ICP27, confirming the findings that low levels of ICP4 have a stimulatory effect on IE promoters (11, 55).

(ii) **Infections.** To determine whether the patterns of ICP4 localization observed in transfections are detectable during viral infection, ICP4 mutant viruses (n214, n208, n18, n215, d156, and d2) which contain the same mutations as plasmids

FIG. 4. Intracellular localization of wild-type and mutant forms of ICP4 in the presence or absence of ICP27 in transfected cells. Vero cells were cotransfected with pn11 or a mutant ICP4 plasmid (pn6, pn7, pn9, pn10, pΔSac, and pn3Δ28) with or without the plasmid expressing ICP27 (pKHXBH). At 24 h posttransfection, monolayers were processed for IF. Rabbit anti-ICP4 polyclonal antibody (R021) was used as the primary antibody. IF-positive cells were categorized and counted as described in the legend to Fig. 2, except that categories N>C, N=C, and N<C were combined and presented as N+C. The percentages of IF-positive cells that fell into each category and the total number (#) of IF-positive cells examined in each transfection are shown on the right; a diagram indicating the forms of ICP4 expressed by the mutant plasmids is shown on the left. The numbers at the ends of truncated polypeptides represent the amino acids which remain unaltered in the peptides. The designations of the mutant viruses containing the same mutations as in the plasmids are shown in the column on the far left.

pn6, pn7, pn9, pn10, pΔSac, and pn3Δ28, respectively (Fig. 4, left-hand side), were used to infect Vero cells. Figure 5 shows that in KOS-infected cells, ICP4 was localized predominantly but not exclusively to nuclei. Consistent with the results of transfection assays, n214- and d156-infected cells, which express mutant forms of ICP4 lacking the NLS, exhibited ICP4-specific staining only in the cytoplasm (Fig. 5). Although ICP4 was localized predominantly to the cytoplasm in the majority of n215- and d2-infected cells, the diffuse nuclear staining, which was greater than or equal to the cytoplasmic staining, was evident in a small number of these cells. In n208- and n18-infected cells, however, ICP4 was localized exclusively to nuclei. These observations are consistent with the previous findings of DeLuca and Schaffer (13).

The results obtained from infected cells (Fig. 5) confirm and

extend the results obtained in transfected cells (Fig. 4). The ICP4 polypeptides specified by KOS, n215, and d2, containing both the NLS and amino acid residues 820 to 1029, responded to the negative effect of ICP27 in infected cells. Notably, relative to the nuclear localization of wild-type ICP4, the nuclear localization of the mutant forms of ICP4 specified by viruses n215 and d2 or plasmids pn10 and pn3Δ28 was strongly inhibited in infected cells but less strongly inhibited in transfected cells.

As expected, the ICP4 staining pattern was nearly identical in n208-, n18-, and 5dl1.2-infected cells (Fig. 5C, D, and H, respectively), i.e., the pattern of ICP4-specific fluorescence produced by mutant viruses (n208 and n18) lacking the region of the ICP4 molecule responsive to ICP27 was indistinguish-

FIG. 5. Staining patterns of mutant and wild-type ICP4 peptides in virus-infected cells. Vero cells were infected with 20 PFU of KOS or ICP4 mutant viruses (n214, n208, n18, n215, d156, and d2) per cell or with the ICP27 deletion mutant virus, 5dl1.2, or were mock infected as indicated. At 5 h postinfection, monolayers on coverslips were processed for IF. Polyclonal rabbit antibody R021, specific for ICP4, was used as the primary antibody, as described in the legend to Fig. 1.

able from that of a mutant virus lacking functional ICP27 (5dl1.2).

It should be noted that the intranuclear staining patterns of the ICP4 peptides specified by *n208* and *n18* were quite different from that of KOS in that no diffuse globular staining was evident in cells infected with these mutants. This observation is consistent with the DNA-negative phenotypes of *n208* and *n18* (13).

As suggested by the results of transfection assays and confirmed by observations of virus-infected cells, the region of the ICP4 molecule required for its responsiveness to the negative effect of ICP27 lies between amino acid residues 820 and 1029.

Correlation between the localization pattern of ICP4 and the level of expression and localization pattern of ICP27. The negative effect of ICP27 on the nuclear localization of ICP4 has been shown in infected cells (Fig. 1) and in transfected cells (Fig. 2). We next attempted to determine whether the localization pattern of ICP4 could be correlated with the level of expression and localization pattern of ICP27.

For this purpose, a double-staining experiment was performed on cells cotransfected with plasmids expressing ICP4 and ICP27 (Table 1). This experiment permits one to examine the expression levels and localization patterns of ICP4 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 ICP4 and ICP27. Twenty-four hours after transfection, cells were processed for IF as described in Materials and Methods. In interpreting the results of this test, 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. In cotransfection experiments, the intensity of ICP27 staining when present only in the nucleus was also weak but when present in both nucleus and cytoplasm was considerably stronger.

The following general observations were made from these cotransfection, double-staining experiments (Table 1). ICP4 was detected exclusively in the nucleus of less than half (38%) of the ICP4-positive cells examined. In two-thirds of these cells, ICP27 was not detected, 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 ICP4. In approximately half (51%) of the ICP4-positive cells examined, ICP4 was detected either predominantly in the nucleus or equally in the nucleus and cytoplasm. ICP27 was detected in all of these cells, a significant cytoplasmic component being present in most of the cells (89%) and an exclusively nuclear component in only 11% of the cells. Very few (11%) ICP4-positive cells exhibited predominantly cytoplasmic staining. In all of these cells, a significant cytoplasmic component of ICP27 staining was present. These findings indicate that the cytoplasmic localization of ICP27 corresponds closely with the cytoplasmic localization of ICP4.

The double-staining experiment just described demonstrated a relationship between the localization pattern of ICP4 and the expression level and localization pattern of ICP27 in transfected cells. We next attempted to determine whether a similar correlation could be demonstrated in infected cells. Recall that in the absence of the repressing activity of ICP4, all IE proteins, including ICP27, are overexpressed (10, 13, 14). Therefore, we reasoned that if the expression level of ICP27 is indeed a critical factor, the pattern of ICP4 localization in cells infected with *n215* or *d2* should be dependent on the multiplicity of infection (i.e., dependent on the level of expression of ICP27). Thus, in this test, ICP4 should be localized predomi-

TABLE 1. Localization of ICP4 and ICP27 in cells cotransfected with plasmids expressing wild-type ICP4 and ICP27 and doubly stained for ICP4 and ICP27

Staining or expression pattern ^a	No. of ICP4-positive cells (%) with staining or expression pattern:	
	ICP4 staining pattern	ICP27 expression pattern
N staining	86 (38)	
Expression patterns		
O		57 (66)
N		29 (34)
N≥C		0 (0)
N<C		0 (0)
N≥C staining	114 (51)	
Expression patterns		
O		0 (0)
N		12 (11)
N≥C		102 (89)
N<C		0 (0)
N<C staining	24 (11)	
Expression patterns		
O		0 (0)
N		0 (0)
N≥C		13 (54)
N<C		11 (46)

^a Staining patterns: 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. Expression patterns: O, not expressing ICP27; N, expressing ICP27 exclusively in the nucleus; N≥C, expressing ICP27 in the nucleus to a greater extent than or equal to the cytoplasm; N<C, expressing ICP27 in the nucleus less than in the cytoplasm.

nantly to the nuclei of cells infected with *n215* or *d2* at low multiplicities of infection and to both nuclei and cytoplasm at higher multiplicities of infection. To test the effects of ICP27 concentration during infection, Vero cells were infected with *n208*, *n18*, *n215*, or *d2*, at multiplicities of infection of 0.5, 5, and 20 PFU per cell, and the localization of ICP4 was examined (Fig. 6). The ICP27 deletion mutant virus, *5dl1.2*, and KOS were included as controls at each multiplicity of infection tested. The results of these tests demonstrated the following. (i) In cells infected with *n215* and *d2*, the lower the multiplicity of infection, the greater the number of ICP4-positive cells that exhibited exclusively nuclear staining. Thus, at a multiplicity of infection of 0.5 PFU per cell, more than 80% of ICP4-positive cells exhibited exclusively nuclear staining (Fig. 6G and J). In contrast, the higher the multiplicity of infection of *n215* and *d2*, the greater the number of cells exhibiting a cytoplasmic component of ICP4 staining (Fig. 6G, H, and I, or Fig. 6J, K, and L). (ii) At all multiplicities of infection tested, ICP4 was always localized exclusively to the nuclei of *5dl1.2*-, *n208* (data not shown, but see Fig. 5H and C for multiplicity of infection of 20 PFU per cell)-, and *n18* (Fig. 6D, E, and F)-infected cells. (iii) At all multiplicities of infections of KOS tested, ICP4 was always localized predominantly to nuclei, a lesser proportion being present in the cytoplasm (Fig. 6A, B, and C). However, it should be noted that more cells with strong globular, nuclear, ICP4-specific staining and weak diffuse cytoplasmic staining were present with higher multiplicities of KOS infection. This multiplicity-dependent phenotype was more prominent in *n215*- and *d2*-infected cells, which was likely due to the absence of down-regulation of ICP27 expression by ICP4 in cells infected with ICP4 mutant viruses.

FIG. 6. Staining pattern of ICP4 in cells infected with 0.5, 5, or 20 PFU of KOS, *n18*, *n215*, or *d2* per cell. Vero cells were infected with KOS, *n18*, *n215*, or *d2* at the multiplicities of infection (MOI) indicated and were processed at 5 h postinfection for IF with polyclonal anti-ICP4 antibody R021, as described in the legend to Fig. 1.

Although the results of these studies suggested that the concentration of ICP27 is critical in determining the intracellular staining pattern of ICP4, the correlation between the localization pattern of ICP4 and the expression level and localization pattern of ICP27 within a given infected cell had not been established definitively. To address this problem more definitively, Vero cells were infected with ICP4 mutants *n18* (the *n18* form of ICP4 does not respond to ICP27), *n215*, or *d2* (both the *n215* and *d2* forms of ICP4 respond to ICP27) at 5 PFU per cell (Fig. 7). At 5 h postinfection, monolayers were processed for IF, and cells were doubly stained for ICP4 and ICP27. A multiplicity of infection of 5 PFU per cell was chosen for these tests, because all ICP4-specific localization patterns characteristic of these mutants are present at this multiplicity of infection (Fig. 6). In *n215*- and *d2*-infected cells that expressed relatively high levels of ICP27 in both the nucleus and cytoplasm (Fig. 7D and F, cells with arrows), ICP4 was detected either predominantly in the cytoplasm or equally in the cytoplasm and the nucleus (Fig. 7C and E, cells with arrows). In contrast, in cells in which ICP27 staining was weak and predominantly nuclear (Fig. 7D and F, cells with arrowheads), ICP4 was also found predominantly in the nucleus (Fig. 7C and E cells with arrowheads). In *n18*-infected cells, ICP4 was always detected exclusively in the nucleus of every ICP4-positive cell, whether ICP27 staining was intense or weak in infected cells (Fig. 6A and B). In this experiment, we also included KOS as a control. In KOS-infected cells, staining of ICP4 and ICP27 was evident predominantly in the nucleus, however, ICP4 was present in replication compartments (Fig. 6) and the intensity of ICP27 staining in these cells was generally weaker than in ICP4 mutant-infected cells (e.g., *n18*, *n215*, and *d2*) (data not shown).

Collectively, the data presented in Table 1 and Fig. 6 and 7 indicate that the level of ICP27 expression and the localization pattern of ICP27 are critical for its inhibitory effect on the nuclear localization of ICP4 in transfected and infected cells.

DISCUSSION

We have shown previously that ICP27 has an inhibitory effect on the nuclear localization of ICP0 in infected cells (62). In this study, we have demonstrated that ICP27, but not ICP0 or ICP22, has an inhibitory effect on the nuclear localization of ICP4 in infected cells. Studies of transfected cells have confirmed these findings, indicating that the localization patterns of ICP4 and ICP0 are regulated primarily by the IE proteins themselves.

The negative effects of ICP27 and ICP0 on the nuclear localization of ICP4 are distinct. The observations that the staining pattern of ICP4 in wild-type virus-infected cells is dependent upon the multiplicity of infection (Fig. 6A, B, and C) and upon the time postinfection (28) suggest that the level of expression of ICP4 is an important determinant of its localization within cells. The results of transfection assays suggest a relatively minor role for the level of ICP4 expression in determining this pattern (Fig. 2B, experiment 3). Using transient expression assays, we have shown clearly that the inhibitory effect of ICP27 on the nuclear localization of ICP4 is distinct from the ICP4 concentration effect. Further, the localization pattern of ICP4 in cells infected with wild-type virus is different from the pattern seen in cells infected with an ICP27 deletion mutant virus. Although the mechanism by which ICP27 inhibits the nuclear localization of ICP4 is presently unknown, it probably does not involve increased

FIG. 7. Comparison of the staining patterns of ICP4 and ICP27 in ICP4 mutant virus-infected cells. Vero cells were infected with 5 PFU per cell of either *n18*, *n215*, or *d2* and cells were processed for IF at 5 h postinfection as described in the legend to Fig. 1. Cells were doubly stained for ICP4 and ICP27 with a mixture of R021 (rabbit anti-ICP4 polyclonal antibody) and H1113 (mouse anti-ICP27 monoclonal antibody) as primary antibodies. RITC-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit immunoglobulin antibodies were used as secondary antibodies.

expression of ICP4 by ICP27. It has been shown previously that ICP27 does not affect the level of ICP4 expression in transient assays (58) nor does it activate expression of other genes under ICP4 promoter control (36). Moreover, in cells infected with the wild-type and ICP27 deletion mutant viruses at the same multiplicities of infection, the levels of ICP4 expression are approximately the same as determined by immunoprecipitation and by Western immunoblot analysis (unpublished results) (32). Collectively, the observations reported in this study and observations cited previously allow us to conclude that ICP27 inhibits the nuclear localization of ICP4 by a mechanism that does not involve enhancement of ICP4 expression.

As shown in Fig. 2B, ICP0 may have a modest inhibitory effect on the nuclear localization of ICP4. However, no distinction could be found between the localization pattern of ICP4 in cells transfected with a plasmid expressing ICP4 alone and the pattern seen in cells cotransfected with the plasmids expressing ICP0 and ICP4. Because ICP0 can enhance expression from the ICP4 promoter in transient assays (4) and in infected cells (5), we suggest that the inhibitory effect of ICP0 on the nuclear localization of ICP4 likely results from the enhancing effect of ICP0 on the expression of ICP4.

Inhibitory effects of ICP27 on the nuclear localization of wild-type and mutant forms of ICP4 in transfected and infected cells. The region of the ICP4 molecule that responds to the negative effect of ICP27 was mapped in this study to the C-terminal portion of the protein between amino acid residues 820 and 1029. Notably, however, among the different forms of ICP4 specified by plasmids pn11, pn10, and pn3Δ28 and their corresponding viruses (KOS, *n215*, and *d2*, respectively)—all of which contain the ICP27-responsive region—the ability of ICP27 to inhibit nuclear localization varied considerably. This observation suggests that secondary configuration and other regions of ICP4 are also likely important for responsiveness to ICP27.

The nuclear localization of mutant forms of ICP4 specified by plasmids pn10 and pn3Δ28 in transient expression assays was inhibited less strongly than the same forms of ICP4 specified by the corresponding mutant viruses, *n215* and *d2*, during viral infection. We interpret this finding to suggest that in addition to ICP27, other IE proteins expressed in ICP4 mutant virus-infected cells also affect the nuclear localization of ICP4. Indeed, the observation that ICP0 alone had little or no inhibitory effect on the nuclear localization of ICP4 but dramatically enhanced the inhibitory effect of ICP27 on the nuclear localization of ICP4 in transient expression assays (Fig. 2B, experiments 1 and 2) supports this hypothesis. Moreover, the ability of ICP0 to enhance the expression of ICP27 (our unpublished data) and the activity of the ICP27 promoter (55) in transient expression assays indicate that ICP0 likely enhances the inhibitory effect of ICP27 during viral infection by enhancing the expression level of ICP27. In the same vein, the virion-associated transactivator, VP16, likely enhances the inhibitory effect of ICP27 in infected cells by enhancing the expression level of ICP27. Furthermore, we suggest that wild-type ICP4, like ICP0, can enhance the inhibitory effect of ICP27 on its own nuclear localization in transient expression assays but represses the inhibitory effect of ICP27 in infected cells on the basis of (i) the ability of low levels of ICP4 to activate ICP27 expression in transfected cells (this study) and (ii) the ability of low levels of ICP4 to enhance the activity of IE promoters in transient expression assays (11, 55).

A possible mechanism by which ICP27 regulates the localization of ICP4. In light of the fact that the C terminus of ICP27 was shown to be required for its inhibitory effect on the nuclear localization of ICP4 (this study) and ICP0 (62), the same mechanism may be involved in inhibiting the nuclear localization of both proteins. Although the mechanism by which ICP27 affects the nuclear localization of these two proteins is unknown, ICP0, ICP4, and ICP27 are nuclear phosphoproteins, and phosphate has been shown to cycle on and off each of these proteins during the course of viral infection (1, 44, 60). Consequently, the possibility that phosphorylation determines the activities and intracellular localization patterns of these IE proteins is intriguing. Studies of ICP4 have suggested a role for phosphorylation in modulating the activity, electrophoretic mobility, and intracellular localization pattern of ICP4 (1, 14, 37, 43, 44, 60). Of special significance to this study is the ability of ICP27 to alter the electrophoretic mobility of ICP4 in both infected and transfected cells (36, 49, 58). The ability of ICP27 to affect the electrophoretic mobility of ICP4 is thought to be due to differences in phosphorylation (61), leading to the speculation that ICP27 may possess kinase or phosphatase activity. Although this is an attractive hypothesis, no such activity has yet 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, and thus indirectly regulate the intracellular localization of ICP0 and ICP4.

Our previous study documented the ability of ICP4 and ICP27 to regulate the intracellular localization of ICP0 (62). This study provides a second example of such regulation in which ICP27 and ICP0 affect the intracellular localization of ICP4, further confirming the complex cooperativity among these proteins in determining their localization within infected cells. These two examples of cooperativity imply that regulation of gene expression in HSV probably involves compartmental constraints, i.e., the intracellular locations of ICP0, ICP4, and ICP27 and their association with specific cellular structures and compartments are likely significant factors in determining their abilities to perform their well-documented

regulatory functions. The numerous functional implications of this mode of regulation have yet to be explored.

The relationship between the intracellular locations and functions of viral regulatory proteins has not been well studied. However, the activities of several cellular regulatory proteins have been correlated with their intracellular localization (22). For example, the intracellular localization of the *Saccharomyces cerevisiae* transcription factor SWI5, is cell cycle dependent (40). This protein is found in the nucleus of cells in G₁ but is cytoplasmic in cells in other phases of the cell cycle. The intracellular localization of the ubiquitous mammalian transcription factor NF- κ B, is regulated by a cytoplasmic inhibitory protein, I κ B. I κ B retains NF- κ B in the cytoplasm through a direct interaction with it (3, 21). In both cases, nuclear localization is required for activity. Despite these interesting results, progress in studies designed to elucidate the molecular basis for the relationship between location and function has been slow because of difficulties associated with the genetic and biochemical manipulation of cellular genes and proteins. Because of the ease with which HSV can be manipulated genetically and biochemically, this virus offers an especially useful eukaryotic model for investigating the relationships between intracellular location and function.

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