Overexpression of the Herpes Simplex Virus Type 1 Immediate-Early Regulatory Protein, ICP27, Is Responsible for the Aberrant Localization of ICP0 and Mutant Forms of ICP4 in ICP4 Mutant Virus-Infected Cells

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ICP0 and ICP4 are immediate-early regulatory proteins of herpes simplex virus type 1. Previous studies by Knipe and Smith demonstrated that these two proteins are characteristically observed in the nuclei of wild-type virus-infected cells but predominantly in the cytoplasm of cells infected with several ICP4 temperature-sensitive (*ts*) mutant viruses at the nonpermissive temperature (NPT) (D. M. Knipe and J. L. Smith, *Mol. Cell. Biol.* 6:2371–2381, 1986). Consistent with this observation, it has been shown previously that ICP0 is present predominantly in the cytoplasm of cells infected with an ICP4 null mutant virus (*n12*) at high multiplicities of infection and that the level of ICP27, a third viral regulatory protein, plays an important role in determining the intracellular localization of ICP0 (Z. Zhu, W. Cai, and P. A. Schaffer, *J. Virol.* 68:3027–3040, 1994). To address whether the cytoplasmic localization of ICP0 is a common feature of cells infected with all ICP4 mutant viruses or whether mutant ICP4 polypeptides, together with ICP27, determine the intracellular localization of ICP0, we used double-staining immunofluorescence tests to examine the intracellular staining patterns of ICP0 and ICP4 in cells infected with an extensive series of ICP4 mutant viruses. In these tests, compared with the localization pattern of ICP0 in wild-type virus-infected cells, more ICP0 was detected in the cytoplasm of cells infected with all ICP4 mutants tested at high multiplicities of infection. Each of the mutant forms of ICP4 exhibiting predominantly cytoplasmic staining contains both the nuclear localization signal and the previously mapped ICP27-responsive region (Z. Zhu and P. A. Schaffer, *J. Virol.* 69:49–59, 1995). No correlation between the intracellular staining patterns of ICP0 and mutant forms of ICP4 was demonstrated, suggesting that mutant ICP4 polypeptides per se are not responsible for retention of ICP0 in the cytoplasm. This observation was confirmed in studies of cells cotransfected with plasmids expressing ICP0 and mutant forms of ICP4, in which the staining pattern of ICP0 was not changed in the presence of mutant ICP4 proteins. Studies of cells infected at low multiplicities with a variety of ICP4 *ts* mutant viruses at the NPT showed that both ICP0 and *ts* forms of ICP4 were localized predominantly within the nucleus. These observations are a further indication that the aberrant localization of the *ts* forms of ICP4 at the NPT is not a direct result of specific mutations in the ICP4 gene. In the final series of tests, the localization of ICP0 in cells infected with a double-mutant virus unable to express either ICP4 or ICP27 was examined. In these tests, ICP0 was detected exclusively in the nuclei of Vero cells but in both the nuclei and the cytoplasm of ICP27-expressing cells infected with the double mutant. These results demonstrate that ICP27, rather than the absence of functional ICP4, is responsible for the cytoplasmic localization of ICP0 in ICP4 mutant virus-infected cells. Taken together, these findings demonstrate that the aberrant localization of ICP0 and certain mutant forms of ICP4 in cells infected with ICP4 mutant viruses is mediated by high levels of ICP27 resulting from the inability of mutant forms of ICP4 to repress the expression of ICP27.

Five virus-specified immediate-early (IE) genes are expressed in cells shortly after infection with herpes simplex virus type 1 (HSV-1). Expression of early (E) viral genes, synthesis of viral DNA, and expression of late (L) viral genes follow and are dependent upon IE gene expression. Four of the five IE proteins, infected-cell proteins ICP0, ICP4, ICP22, and ICP27, are recognized regulatory proteins. All four are phosphory-

lated and localized predominantly to the nuclei of wild-type virus-infected cells (1, 28, 42). As the major transcriptional regulatory protein of HSV-1, ICP4 activates E and L gene expression and represses its own expression, as well as that of other IE genes (7–9, 12, 13, 15, 16, 23, 26, 27, 31). ICP4 has a stimulatory effect on IE gene expression at low concentrations but an inhibitory effect at high concentrations (9). Recent studies have suggested that ICP4 exerts both its activating and its repressing activities through a mechanism involving interactions with general transcription factors, including TBP and TFIIB (18, 39). Although ICP0 is not essential for viral replication, it is able to activate expression of all three kinetic classes of viral genes (IE, E, and L) alone and can activate E

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and L gene expression synergistically with ICP4 (4, 5, 13–16, 23, 26, 31). ICP27 by itself has little effect on HSV gene expression (14, 38) but is able to further enhance or repress the transactivating activities of ICP4 and ICP0 (14, 25, 32, 33, 38, 40). ICP22 modifies the carboxyl-terminal domain of RNA polymerase II during productive infection (34, 35) and is required for efficient viral replication in some cell types in vitro and in vivo (2, 37).

As summarized above, available evidence indicates that the activities of individual IE proteins and cooperative interactions among these proteins play key roles in regulating HSV-1 gene expression. The manner in which the activities of these proteins are regulated and the molecular basis for the functional cooperativity among them are not understood. Several mechanisms have been proposed as a basis for functional cooperativity. These include direct protein-protein interactions, as well as posttranslational modification, transcriptional and posttranscriptional control, and compartmental constraints of one IE protein by another. With regard to compartmental constraints, Knipe and Smith (21) made the interesting observation that the nuclear localization of ICP0 characteristic of wild-type virus-infected cells is aberrant in cells infected with ICP4 temperature-sensitive (*ts*) mutant viruses at the nonpermissive temperature (NPT) in that ICP0 was detected predominantly in the cytoplasm. These authors also noted that *ts* forms of ICP4 were also cytoplasmic in these cells (wild-type ICP4 is predominantly nuclear). Although the aberrant localization of ICP0 and mutant forms of ICP4 in ICP4 mutant virus-infected cells is clear from these studies, the molecular basis for this altered localization has not been examined.

Consistent with the observations of Knipe and Smith, we have observed that the nuclear localization of ICP0 is inhibited in cells infected at high multiplicities of infection with an ICP4 nonsense mutant, *n12*, and in cells cotransfected with plasmids expressing ICP0 and ICP27 (43). Furthermore, we have shown that the high level of ICP27 (resulting from the absence of the repressive activity of ICP4 on IE genes), rather than the *n12* form of ICP4 itself, is responsible for the altered localization of ICP0 in *n12*-infected cells. In addition, we have shown that the nuclear localization of the altered forms of ICP4 which contain an intact nuclear localization signal (NLS) and overexpress ICP4 is also predominantly cytoplasmic in cells infected at high multiplicities with ICP4 mutant viruses and that, as for ICP0, ICP27 plays a critical role in determining the localization of ICP4 (44). The region of ICP4 that responds to the inhibitory effect of ICP27 was mapped to the C terminus of the protein, between amino acid residues 820 and 1029 (44).

Despite these observations, it remained unclear whether the cytoplasmic localization of ICP0 characteristic of *n12*-infected cells is also characteristic of cells infected with other ICP4 mutant viruses. The *n12* mutant virus is considered to be null for ICP4 because the ICP4 peptide specified by this virus is very small (it consists of the 251 N-terminal amino acid residues, or about one-fifth of the wild-type ICP4 protein) and because it exhibits no detectable transactivating or DNA binding activity (11). For this reason, *n12* is not the most appropriate mutant virus with which to study the effect of mutant forms of ICP4 on the nuclear localization of ICP0. To determine whether the aberrant localization of ICP0 is a common feature of cells infected with ICP4 mutant viruses and to ascertain what roles mutant ICP4 polypeptides and ICP27 may play in determining the intracellular localization of ICP0 and ICP4, we examined the staining patterns of ICP0 and ICP4 in cells infected with a variety of *ts*, nonsense, deletion, and spontaneous mutants of ICP4 by double-staining immunofluorescence (IF) tests. Notably, ICP0 exhibited aberrant localization

in cells infected with all of the ICP4 mutant viruses tested at high but not at low multiplicities of infection. Furthermore, the nuclear localization of all *ts* forms of ICP4 and certain other mutant forms of ICP4 was also inhibited in these cells. The results of these studies lead us to conclude that the aberrant localization of ICP0 and ICP4 is not due to mutant ICP4 peptides per se but to high levels of ICP27 which are synthesized in the absence of the repressing activity of ICP4 for IE genes in ICP4 mutant virus-infected cells.

MATERIALS AND METHODS

Cells and viruses. Vero cells were used in all transfections and most infections. The ICP27-expressing cell line, 3-3, and the ICP4-expressing cell line, E5, both derived from Vero cells, were used to propagate and assay ICP27 and ICP4 mutant viruses, respectively (10, 24). All 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. The ICP27 deletion mutant virus, *5dl1.2* (24), and the ICP0 null mutant 7134 (4), both derived from strain KOS, and a large number of KOS-derived ICP4 mutant viruses (7, 8, 11, 12), including nonsense mutants (*n12*, *n214*, *n208*, *n18*, and *n215*), deletion mutants (*d120*, *d156*, and *d2*), and *ts* mutants (*tsB2*, *tsB21*, *tsB32*, *tsL14*, and *ts48*), were included in this study. Other ICP4 *ts* mutant viruses, *ts756* (19), derived from strain KOS1.1, and *tsK* (29, 30), derived from strain 17, were kindly provided by M. Levine (University of Michigan) and C. Preston (MRC Virology Unit, Glasgow, Scotland), respectively. The titers of infectious virus in stocks of all the *ts* mutant viruses were determined simultaneously on Vero cell monolayers at 34°C, and stocks of nonsense, deletion, and other mutant viruses were titrated on monolayers of the appropriate cell lines at 37°C. The ICP4-ICP27 double-mutant virus, *d92*, was derived by recombination between *d120* and *5dl1.2* (36). Thus, *d92* contains the *d120* mutant ICP4 allele at both ICP4 loci and the *5dl1.2* mutant ICP27 allele at the single ICP27 locus and was propagated in the E26 cell line, which provides comparable levels of ICP4 and ICP27.

Plasmids. The plasmids used in transfections include the following: (i) plasmid *pn11*, which contains the wild-type ICP4 gene from strain KOS (10); (ii) ICP4 nonsense and deletion mutant plasmids *pn6*, *pn7*, *pn9*, *pn10*, *pΔSac*, and *pn3Δ28*, provided by N. DeLuca (10, 11) (these plasmids contain the same mutations as those in KOS-derived mutant viruses *n214*, *n208*, *n18*, *n215*, *d156*, and *d2*, respectively [11]); and (iii) plasmid *pSH*, which contains the wild-type ICP0 gene from strain KOS (4).

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). Rabbit polyclonal antibody J17, which is directed against ICP0, has been described previously (43). Rabbit polyclonal antibody R021, which is directed against ICP4 (6), 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 (immunoglobulin G [IgG] plus IgA plus IgM) antibodies were obtained from Cappel Laboratories (Durham, N.C.).

IF tests. Vero cells and 3-3 cells were grown on sterile glass coverslips in 60-mm-diameter petri dishes. At 24 h postseeding, the cells were infected with wild-type or mutant viruses at the multiplicity of infection and temperature indicated in each figure legend. Alternatively, cells were transfected with plasmid DNAs by the calcium phosphate precipitation procedure as previously described (43). The combinations of the plasmids transfected are indicated below. 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 has been described previously (43). Cells were stained to detect ICP0 by using mouse monoclonal antibody H1083 (1:800) followed by RITC-conjugated goat anti-mouse immunoglobulin as the second antibody (1:300). To detect ICP0 and ICP4, ICP0 and ICP27, or ICP4 and ICP27 simultaneously, cells were double stained with a mixture of primary antibodies H1083 (1:800) and R021 (1:150), J17 (1:50) and H1113 (1:600), or R021 (1:150) and H1113 (1:600), respectively, followed by a mixture of secondary antibodies including RITC-conjugated goat anti-mouse (1:300) and FITC-conjugated goat anti-rabbit (1:200) immunoglobulin antibodies.

Monolayers were viewed with a Zeiss fluorescence microscope with a 40×/1.3, oil, PH3, Plan-Neofluar objective, as described previously (43). Photographs were taken with Kodak Tri-X pan 400 film.

Immunoblot analysis. Vero and 3-3 cells were infected as described above and in the legend to Fig. 6. At 5 h postinfection, cells were washed with Tris-buffered saline (TBS) (137 mM NaCl, 5 mM KCl, 25 mM Tris-HCl; pH 7.4) and lysed with sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.0025% bromophenol blue) containing 1 mM leupeptin (Sigma, St. Louis, Mo.) to inhibit proteinase activities present in 3-3 and Vero cell extracts. Protein samples were boiled at 100°C for 5 min and then separated by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (22) as modified by Gibson and Roizman (17). The sepa-

rated proteins were transferred to a nitrocellulose filter by electroblotting (3, 41). ICP27 was detected by treatment of the filter with mouse monoclonal antibody H1113 (1:1,000 dilution) followed by an alkaline phosphatase detection system (Bio-Rad Laboratories, Hercules, Calif.). This procedure ensured that the results of immunoblotting were a true reflection of the consequence of infection. Thus, equal numbers of cells were used in each infection, cells were harvested and lysates were prepared identically for each culture, and equal portions of each lysate were loaded on the gel.

RESULTS

ICP0 and ICP4 are present in the cytoplasm rather than the nuclei of cells infected with ICP4 *ts* mutant viruses at the NPT. To examine the localization patterns of ICP0 and ICP4 in cells infected with ICP4 *ts* mutants, Vero cells were infected with 20 PFU of *tsK*, *ts756*, *ts48*, *tsB2*, *tsB21*, *tsB32*, or *tsL14* per cell at the nonpermissive (39.6°C) and permissive (34°C; control) temperatures. As viral controls, Vero cells were mock infected or infected at 34°C with 20 PFU of the wild-type virus, KOS, the ICP4 null mutant virus, *n12*, or the ICP27 null mutant virus, *5dl1.2* per cell. The cells were double stained for ICP0 and ICP4 as described above. The results of these tests are shown in Fig. 1.

The appearance of ICP0, ICP4, and ICP27 staining was uniform throughout these studies: ICP0 and ICP4 always produced fine granular or punctate staining. The staining for ICP27 was more diffuse (data not shown). In KOS-infected cells, both ICP0 and ICP4 were detected predominantly in the nuclei, whereas in *5dl1.2*-infected cells, these proteins were detected exclusively within the nuclei (compare Fig. 1A and D with B and E). In contrast, ICP0 and ICP4 were localized predominantly in the cytoplasm of all ICP4 *ts* mutant-infected cells at 39.6°C (Fig. 1G to N, P, and Q). (Results with *ts48* and *tsB2* are not shown but were identical to those shown in Fig. 1 for other *ts* mutants.) At 34°C, the staining patterns of ICP0 and ICP4 were indistinguishable from the patterns seen in wild-type virus-infected cells, i.e., the proteins were detected predominantly in the nuclei (not shown). As in the case of the *ts* mutants, ICP0 and the mutant form of ICP4 specified by *n12* were localized predominantly in the cytoplasm (Fig. 1O and R). No ICP0- or ICP4-specific staining was detected in mock-infected cells (Fig. 1C and F). Thus, predominantly cytoplasmic rather than nuclear staining patterns of ICP0 and ICP4 are characteristic of cells infected with ICP4 *ts* mutants at the NPT. Because ICP4 and ICP0 are both located in the cytoplasm of mutant virus-infected cells, it is possible that *ts* forms of ICP4 interact with ICP0, thereby retaining ICP0 in the cytoplasm as initially suggested by Knipe and Smith (21).

The localization of ICP0 characteristic of wild-type virus-infected cells is aberrant in cells infected with ICP4 nonsense and deletion mutant viruses. To determine whether the localization of ICP0 in the cytoplasm rather than the nucleus is a common characteristic of cells infected with other kinds of ICP4 mutant viruses, Vero cells were infected with 20 PFU of a variety of ICP4 nonsense and deletion mutants per cell. The mutants tested included nonsense mutants *n12*, *n214*, *n208*, *n18*, and *n215* and deletion mutants *d120*, *d156*, and *d2*. The infected cells were double stained for ICP0 and ICP4, and the results of tests with selected mutant viruses (*n214*, *n208*, *n18*, *n215*, *d156*, and *d2*) are shown in Fig. 2. Results of tests with KOS and *n12* are shown in Fig. 1A and D and 1O and R, respectively.

In cells infected with each of the ICP4 nonsense and deletion mutant viruses, the nuclear localization of ICP0 characteristic of wild-type virus-infected cells (Fig. 1A) was altered, and the degree and nature of the alteration varied from virus to virus. The predominantly nuclear staining pattern charac-

teristic of ICP0 in wild-type virus-infected cells (Fig. 1A) was altered in that staining was more cytoplasmic in *n208*-, *n18*-, and *d156*-infected cells (Fig. 2B, C, and H) than that seen for wild-type virus and staining was predominantly cytoplasmic in cells infected with *n214*, *n215*, or *d2* (Fig. 2A, G, and I). The mutant forms of ICP4 specified by *n214*, *d156*, and *n12* lack the NLS; consequently, these ICP4 polypeptides were localized to the cytoplasm as anticipated (for *n214* and *d156*, see Fig. 2D and K; for *n12*, see Fig. 1R). The mutant forms of ICP4 specified by *n208* and *n18* (Fig. 2E and F) which contain the NLS were localized exclusively to the nucleus. Mutant forms of ICP4 specified by *n215* and *d2* (Fig. 2J and L) also contain the NLS but were localized predominantly, but not exclusively, to the cytoplasm.

Collectively, these observations demonstrate that the nuclear localization of ICP0 was impaired in all ICP4 mutant virus-infected cells and that the localization of ICP0 did not always correlate with the intracellular localization of mutant forms of ICP4. These observations suggest that the hypothesis of Knipe and Smith, that mutant forms of ICP4 interact physically with ICP0, thereby retaining it in the cytoplasm, is not likely the case.

Mutant forms of ICP4 do not inhibit the nuclear localization of ICP0 in transfected cells. To determine whether the findings made with infected cells could be confirmed under circumstances in which cells contained only ICP0 and ICP4 (i.e., in the absence of other viral proteins), Vero cells were transfected either with a plasmid expressing wild-type ICP0 (pSH) alone or with pSH and a plasmid expressing either wild-type ICP4 (pn11) or a mutant form of ICP4 (pn6, pn7, pn9, pn10, pΔSac, and pn3Δ28; these plasmids contain the same mutations as viruses *n214*, *n208*, *n18*, *n215*, *d156*, and *d2*, respectively). The cells were double stained for ICP0 and ICP4, and those exhibiting ICP0-specific fluorescence were counted and classified into one of three groups: cells that exhibited only nuclear ICP0 staining (N), cells that exhibited only cytoplasmic staining (C), or cells that exhibited both nuclear and cytoplasmic staining (N + C) (Table 1). The location patterns of ICP4 in cells exhibiting both ICP0- and ICP4-specific fluorescence are also presented in Table 1.

ICP0 was detected predominantly in the nuclei of transfected cells in the absence of other viral proteins. Notably, none of the mutant forms of ICP4 had a major effect on the nuclear localization of ICP0 in cotransfected cells, although wild-type ICP4 (pn11) had a modest enhancing effect on the nuclear localization of ICP0, as was observed previously (43). The slight enhancing effects of the mutant forms of ICP4 specified by pn7 and pn9 on the nuclear localization of ICP0 may be a function of the partial regulatory activities exhibited by these polypeptides (10), suggesting the possibility that down-regulation of ICP0 expression by wild-type or certain mutant forms of ICP4 may promote the nuclear localization of ICP0. The regulatory effects of the pn7 and pn9 forms of ICP4 may also correlate with the reduced impairment of the nuclear localization of ICP0 in cells infected with the corresponding mutant viruses, *n208* and *n18* (Fig. 2B and C).

Notably, in cells cotransfected with pSH and pn7 or pn9, in which mutant forms of ICP4 were detected almost exclusively in the nuclei, ICP0 was also detected predominantly in the nuclei. In contrast, in cells cotransfected with pSH and pn6 or pΔSac, in which mutant forms of ICP4 were detected primarily in the cytoplasm, ICP0 was again detected predominantly in the nuclei. On the basis of these observations, no correlation existed between the intracellular compartmentalization of ICP0 and ICP4 in transfected cells. Also, no difference between the localization patterns of mutant forms of ICP4 in the

FIG. 1. Staining patterns of ICP0 and ICP4 in wild-type and mutant virus-infected cells. Vero cells were mock infected or infected with 20 PFU of the following viruses per cell: ICP4 *ts* mutant viruses *tsK*, *ts756*, *tsB21*, *tsB32*, or *tsL14*; the ICP4 null mutant virus *n12*; the ICP27 null mutant virus *5dl1.2*; and wild-type virus strain KOS. Infections were carried out at 39.6°C (the NPT) for ICP4 *ts* mutants and at 37°C for all other viruses. At 5 h postinfection, cells were processed for IF and were double stained for ICP0 and ICP4 with H1083 (mouse anti-ICP0 monoclonal antibody) and R021 (rabbit anti-ICP4 polyclonal antibody) as primary antibodies and with RITC-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit immunoglobulin antibodies as secondary antibodies. ICP0-specific staining (A, B, C, G, H, I, M, N, and O) and ICP4-specific staining in the same cells (D, E, F, J, K, L, P, Q, and R, respectively) are shown.

absence of ICP0 (44) or in the presence of ICP0 (this study) was noted, suggesting that ICP0 has no significant effect on the localization of wild-type or mutant forms of ICP4 in transfected cells. These observations are consistent with the previous finding that ICP0 has no effect on the nuclear localization of ICP4 in infected cells (43).

These findings demonstrate that the mutant forms of ICP4

specified by plasmids pn6, pn7, pn9, pn10, pΔSac, and pn3Δ28 have no effect on the intracellular localization of ICP0 in transfected cells and suggest by extension that the mutant forms of ICP4 specified by viruses *n214*, *n208*, *n18*, *n215*, *d156*, and *d2*, which contain the same mutations as the corresponding plasmids, have no inhibitory effect on the nuclear localization of ICP0 in infected cells.

FIG. 2. Staining patterns of ICP0 and mutant forms of ICP4 in ICP4 mutant virus-infected cells. Vero cells were infected at 37°C with 20 PFU of *n214*, *n208*, *n18*, *n215*, *d156*, or *d2* per cell. At 5 h postinfection, cells were processed for IF and double stained for ICP0 and ICP4 as described in the legend to Fig. 1. ICP0-specific staining (A, B, C, G, H, and I) and ICP4-specific staining in the same cells (D, E, F, J, K, and L, respectively) are shown.

TABLE 1. Localization of ICP0 in cells transfected with a wild-type ICP0-expressing plasmid alone or together with a wild-type or mutant ICP4-expressing plasmid and double stained for ICP0 and ICP4^a

Plasmid ^b	Localization of ICP0 ^c				Properties of ICP4	
	N	N + C	C	Total	Localization	Autoregulation ^d
pSH	69.5	30.5	0	522		
pSH + pn11	86.3	13.7	0	335	N, N > C	+
pSH + pn6	73.2	26.8	0	194	C	—
pSH + pn7	80.2	19.8	0	339	N	+/-
pSH + pn9	76.9	23.1	0	295	N	+/-
pSH + pn10	66.7	33.3	0	249	N, N > C	—
pSH + pΔSac	71.3	28.7	0	265	C	—
pSH + pn3Δ28	67.0	33.0	0	230	N, N > C	—

^a Staining patterns of ICP0 and ICP4 in transfected cells were detected only in the nucleus (N), in both the nucleus and the cytoplasm (N + C), only in the cytoplasm (C), or in the nucleus more than in the cytoplasm (N > C).

^b pSH expresses wild-type ICP0; pn11 expresses wild-type ICP4; and pn6, pn7, pn9, pn10, pΔSac, and pn3Δ28 express mutant forms of ICP4.

^c Percentages of ICP0-positive cells in each category (N, N + C, and C). Total, total number of ICP0-positive cells examined in each transfection.

^d Autoregulatory activity of mutant or wild-type forms of ICP4: —, no activity; +, wild-type activity; and +/-, partial activity (taken from studies of DeLuca and Schaffer [10, 11]).

The localization patterns of ICP0 and certain mutant forms of ICP4 in ICP4 mutant virus-infected cells are altered as a function of the multiplicity of infection. It has been shown previously that the staining pattern of ICP0 in cells infected with the ICP4 null mutant virus, *n12*, became more cytoplasmic as the multiplicity of infection increased (43). To determine whether the localization pattern of ICP0 is multiplicity dependent in cells infected with other ICP4 mutant viruses, Vero cells were infected with a number of ICP4 mutant viruses at multiplicities of 0.5, 5, and 20 PFU per cell and processed for IF at 5 h postinfection. Cells were then double stained for ICP0 and ICP4. The mutant viruses included in this experiment were *n214*, *n208*, *n18*, *n215*, *d156*, *d2*, *tsK*, *ts756*, *ts48*, *tsB2*, *tsB21*, *tsB32*, and *tsL14*. To simplify the presentation of results, only results of representative tests in which cells were infected at 0.5 PFU per cell with *n18*, *n215*, *d156*, and *tsB21* are shown in Fig. 3. Results of tests in which cells were infected at 20 PFU per cell are shown in Fig. 1 and 2. At 5 PFU per cell, localization patterns of ICP0 and ICP4 were intermediate between those at 0.5 and 20 PFU per cell (not shown). The results of tests at all three multiplicities are summarized in Table 2.

On the basis of the results of infections performed with a multiplicity of infection of 20 PFU per cell (Fig. 1 and 2), the ICP4 mutant viruses could be divided into four groups (Table 2) as follows: group 1, *n18* and *n208* (mutant forms of ICP4 specified by these two viruses contain the NLS and were local-

FIG. 3. Staining patterns of ICP0 and mutant forms of ICP4 in ICP4 mutant virus-infected cells. Vero cells were infected at 37°C with 0.5 PFU of *n18* (A and B), *n215* (C and D), or *d156* (E and F) per cell or infected at 39.6°C with 0.5 PFU of *tsB21* per cell (G and H). At 5 h postinfection, the cells were processed for IF and double stained for ICP0 and ICP4 as described in the legend to Fig. 1. ICP0-specific staining (A, C, E, and G) and ICP4-specific staining in the same cells (B, D, F, and H, respectively) are shown.

ized exclusively to the nuclei of infected cells [Fig. 2F and E] [44]); group 2, *n215* and *d2* (mutant forms of ICP4 specified by these two viruses contain the NLS and were localized to both the nuclei and the cytoplasm of infected cells [Fig. 2J and L] [44]); group 3, *n214* and *d156* (mutant forms of ICP4 specified by these two viruses contain no NLS and were localized exclusively to the cytoplasm of infected cells [Fig. 2D and K]); and group 4, all ICP4 *ts* mutants tested. *ts* forms of ICP4 had staining patterns similar to those of mutants in group 2 (compare the staining patterns of *ts* mutants in Fig. 1 with those of *n215* and *d2* in Fig. 2).

In contrast to the more pronounced cytoplasmic staining patterns for ICP0 seen in cells infected with ICP4 mutant viruses at a high multiplicity of infection (20 PFU per cell; Fig. 1 and 2 and Table 2), ICP0 was localized exclusively (*n18*) or predominantly (*n215*, *d156*, and *tsB21*) in the nuclei of most ICP0-positive cells infected at 0.5 PFU per cell (e.g., Fig. 3 and Table 2). At 5 PFU per cell, localization patterns of ICP0 were intermediate between those observed at 20 and 0.5 PFU per cell (Table 2). This observation is consistent with the previous finding that the intracellular localization pattern of ICP0 in cells infected with the ICP4 null mutant virus, *n12* (43), varies as a function of multiplicity.

As for ICP0, the staining patterns of the forms of ICP4 specified by mutant viruses in groups 2 and 4 varied as a

function of multiplicity of infection. At a multiplicity of 20 PFU per cell, the mutant forms of ICP4 specified by *n215* (Fig. 2J) and *tsB21* at the NPT (Fig. 1L) were localized predominantly in the cytoplasm, whereas at a multiplicity of 0.5 PFU per cell, the *n215* (Fig. 3D) and *tsB21* proteins (Fig. 3H) were detected predominantly in the nucleus at the NPT. Similar ICP4 staining patterns were also observed in cells infected with *d2* and all other *ts* mutants tested in this study. Consistent with our previous findings (44), the mutant forms of ICP4 specified by *n18* (Fig. 2F and 3B) and *n208* (Fig. 2E, multiplicity of infection of 20 PFU per cell) were localized exclusively to the nuclei and the mutant forms of ICP4 specified by *d156* (Fig. 2K and 3F) and *n214* (Fig. 2D) (both of which lack the NLS) were localized to the cytoplasm of infected cells at all three multiplicities of infection (Table 2).

These observations demonstrate that the multiplicity dependence of the localization pattern of ICP0 is characteristic of all ICP4 mutant virus-infected cells and thus may also be a function of the ability of the mutant ICP4 protein to repress its own expression. The localization patterns of the forms of ICP4 specified by mutant viruses in groups 2 and 4 also appear to be multiplicity dependent. Notably, each of the mutant forms of ICP4 whose localization pattern depends on the multiplicity of infection contains both the NLS and the previously mapped ICP27-responsive region (44). In light of the ability of *ts* forms

TABLE 2. Localization of ICP4 and ICP0 in cells infected with different groups of ICP4 mutant viruses at different multiplicities of infection

Group	Viruses	Mutation(s) ^a in ICP4	Presence of ICP4 NLS	Protein	Localization of proteins in cells infected at MOI (PFU/ cell) of ^b :		
					20	5	0.5
1	<i>n18, n208</i>	ns	+	ICP4	N	N	N
				ICP0	N > C	N, N > C	N
2	<i>n215, d2</i>	ns, <i>dl</i>	+	ICP4	N ≤ C	N ≥ C	N
				ICP0	N ≤ C	N > C, N	N, N > C
3	<i>d156, n214</i>	<i>dl</i> , ns	–	ICP4	C	C	C
				ICP0	N ≥ C	N > C, N	N, N > C
4	<i>tsB21</i> , etc.	<i>ts</i>	+	ICP4	C, N < C	N ≥ C	N, N > C
				ICP0	N < C	N ≤ C	N, N > C

^a Mutations of ICP4: ns, nonsense; *dl*, deletion.^b Intracellular staining patterns of mutant forms of ICP4 and ICP0 in over 90% of fluorescence-positive (infected cells): N, nuclear; N > C, nuclear greater than cytoplasmic; N < C, nuclear less than cytoplasmic; C, cytoplasmic. The one or two most prominent localization patterns which characterize over 90% of the infected cells are presented in this table; the most prominent pattern is listed first. MOI, multiplicity of infection.

of ICP4 to reach the nucleus at the NPT in cells infected at low multiplicities of infection, we conclude that the *ts* mutations do not affect the ability of the NLS to function under these conditions.

Correlation between the localization patterns of ICP0 and ICP4 and the expression level and localization pattern of ICP27 in ICP4 *ts* mutant-infected cells. Previous studies have shown that ICP27 is able to inhibit the nuclear localization of both ICP0 and ICP4 and that high-level expression of ICP27 can be correlated with the cytoplasmic localization of ICP0 and ICP4 in certain ICP4 mutant virus-infected cells and in cells cotransfected with plasmids expressing either ICP0 or ICP4 and ICP27 (43, 44). To determine whether the level of expression of ICP27 correlates with the cytoplasmic localization of *ts* forms of ICP4 and ICP0 in ICP4 *ts* mutant virus-infected cells at the NPT, Vero cells were infected with ICP4 *ts* mutant virus *tsK*, *ts756*, *ts48*, *tsB2*, *tsB21*, *tsB32*, or *tsL14* at 0.5 PFU per cell and incubated at 39.6°C. The cells were double stained for ICP0 and ICP4, ICP0 and ICP27, and ICP4 and ICP27 at 5 h postinfection. Because similar results were obtained with cells infected with all ICP4 *ts* mutant viruses, only the results of tests with *tsB21*-infected cells are shown in Fig. 4.

In contrast to the predominantly cytoplasmic staining of ICP0 and ICP4 in cells infected with *tsB21* at 20 PFU per cell (Fig. 1I and L), both ICP0 (Fig. 4A) and ICP4 (Fig. 4B and E) were localized either exclusively or predominantly to the nuclei of most cells infected with this mutant at 0.5 PFU per cell, suggesting that the *tsB21* mutation in the ICP4 gene does not affect the function of the NLS as noted above. Moreover, prominent nuclear ICP27-specific staining (Fig. 4D and F) corresponded with a significant cytoplasmic component of ICP0-specific (Fig. 4C) and ICP4-specific (Fig. 4E) staining in *tsB21*-infected cells at the NPT, i.e., in a given cell, when ICP0- or ICP4-specific cytoplasmic staining was evident, ICP27-specific nuclear staining was prominent. Such a correlation between the cytoplasmic localization of ICP0 and ICP4 and expression of ICP27 within a given cell also existed in cells infected with the other six ICP4 *ts* mutant viruses tested (not shown). A similar correlation was also observed between the cytoplasmic localization of ICP0 and prominent expression of ICP27 in cells infected with ICP4 deletion or nonsense mutant viruses, such as the nonsense mutant *n12* (see Fig. 7 of reference 43).

These observations suggest that prominent nuclear expres-

sion of ICP27 in cells infected at the NPT with ICP4 *ts* mutant viruses correlates well with the cytoplasmic localization of ICP0 as seen in cells infected with the ICP4 null mutant virus, *n12* (43).

Overexpression of ICP27, resulting from the absence of functional ICP4, is ultimately responsible for the abnormal localization of ICP0 and ICP4 in ICP4 mutant virus-infected cells. The studies described above led us to conclude that the aberrant localization of ICP0 is a common feature of cells infected with ICP4 mutant viruses and that a close correlation exists between the prominent nuclear expression of ICP27 and cytoplasmic localization of ICP0 and ICP4. It is unclear, however, whether the cytoplasmic localization of ICP0 and ICP4 is a direct effect of a nonfunctional ICP4 or a result of the overexpression of ICP27 which occurs as a consequence of a nonfunctional ICP4. To distinguish between these two possibilities, a double-mutant virus, *d92*, containing mutations in both copies of the ICP4 gene and in the ICP27 gene was used to infect Vero cells or Vero-derived ICP27-expressing cells (3-3 cells). The staining patterns of ICP0 were examined by IF (Fig. 5A to D).

In *d92*-infected 3-3 cells, ICP0 was localized exclusively to the nuclei of the majority (~90%) of cells infected at a multiplicity of infection of 0.5 PFU per cell (Fig. 5A), ~50% of cells infected at 5 PFU per cell (Fig. 5B), and only about 10% of cells infected at 20 PFU per cell (Fig. 5C). In contrast, no matter what the multiplicity of infection, ICP0 was localized exclusively to the nucleus of every *d92*-infected Vero cell (Fig. 5D; 20 PFU per cell). Notably, this staining pattern is identical to that observed in ICP27 null mutant virus (*5dl1.2*)-infected Vero cells (Fig. 1B). Together, these observations indicate that the cytoplasmic localization of ICP0 results from the presence of ICP27, rather than merely the absence of functional ICP4.

To determine whether the multiplicity-dependent phenotype of the ICP0 localization pattern in *d92*-infected cells could be correlated with the level of ICP27 expression in infected cells, 3-3 cells were infected with *d92* at multiplicities of infection of 0.5, 5, and 20 PFU per cell. The cells were stained for ICP0 and ICP27. Figure 5E through G shows the relative intensities of ICP27 expressed in 3-3 cells infected with *d92* at multiplicities of 0.5, 5, and 20 PFU per cell, respectively. The intensity of ICP27-specific staining in 3-3 cells infected with *d92* at 100 PFU per cell (not shown) was similar to that at a multiplicity of infection of 20 PFU per cell (Fig. 5G). On the

FIG. 4. Localization of ICP0, ICP4, and ICP27 in *tsB21*-infected cells at the NPT. Vero cells were infected with 0.5 PFU of *tsB21* per cell and incubated at 39.6°C. At 5 h postinfection, cells were processed for IF and double stained for ICP0 and ICP4 (A and B, respectively), ICP0 and ICP27 (C and D, respectively), and ICP4 and ICP27 (E and F, respectively) as described in Materials and Methods. The proteins examined in each micrograph are indicated adjacent to the micrograph.

basis of the intensity of ICP27-specific staining, the levels of ICP27 expression from the endogenous ICP27 gene increased as the multiplicity increased. Direct measurement of the levels of ICP27 in these cells by immunoblot analysis produced virtually the same results (Fig. 6). No ICP27 was detected in mock-infected 3-3 cells (Fig. 6, lanes 1 and 8), and the levels of ICP27 expression increased as the multiplicities of infection of *d92* (lanes 2 to 5) and *5dl1.2* (lanes 6 and 7) in infected 3-3 cells increased. These observations are consistent with the previous finding that the resident ICP27 gene in 3-3 cells is expressed only following viral infection (24) and demonstrate that the multiplicity dependence of ICP0 localization patterns correlates with both the intensities of ICP27-specific staining and the levels of ICP27 expression in *d92*-infected 3-3 cells. Notably, the level of ICP27 expression remained the same in 3-3 cells infected with *d92* at multiplicities of infection of 20 (Fig. 6, lane 4) and 100 (Fig. 6, lane 5) PFU per cell. This result is consistent with the observation that the intensity of ICP27-specific staining remained the same in 3-3 cells infected with *d92* at multiplicities of 20 (Fig. 5G) and 100 (not shown) PFU per cell and is also consistent with the observation that the percentage of *d92*-infected 3-3 cells exhibiting ICP0 exclusively in the nuclei (~10%) remained the same at a multiplicity of 20 PFU per cell (Fig. 5C) or higher (not shown), suggesting that the level of ICP27 expression in *d92*-infected 3-3 cells reaches a maximum level in infections initiated with 20 PFU per cell and that the percentage of infected cells in which ICP0 is present exclusively in the nuclei is reduced to the minimum as the level of ICP27 expression reaches the maximum. Figure 5H shows the staining pattern of ICP0 in the same cells as shown in Fig. 5F. The degree of ICP0-specific cytoplasmic staining correlated with the staining intensity of ICP27 in the nuclei of individual cells (Fig. 5H and F). The more intense the ICP27 nuclear staining, the more prominent the ICP0 cytoplasmic staining.

In addition to 3-3 cells, the expression levels of ICP27 were also measured in Vero cells infected with 20 PFU of wild-type KOS (Fig. 6, lane 9), *5dl1.2* (lane 10), *d92* (lane 11), ICP0 null mutant 7134 (lane 12), and ICP4 null mutant *n12* (lane 13) per cell. Mutants *5dl1.2*, *d92*, and 7134 and the wild-type virus were chosen as controls for comparing the level of ICP27 expression in *n12*-infected Vero cells. As expected, no ICP27 was detected in cells infected with *5dl1.2* and *d92* because of the deletion of the ICP27 gene in these mutant viruses.

Taken together, these observations demonstrate that the high levels of ICP27 expression in ICP4 mutant virus-infected cells are responsible for the cytoplasmic localization of ICP0 and certain mutant forms of ICP4.

DISCUSSION

ICP0, -4, and -27 are nuclear phosphoproteins that cooperatively orchestrate the expression of HSV-1 genes. In previous studies, it was shown that ICP0 is localized predominantly to the cytoplasm rather than the nuclei of cells infected with the ICP4 null mutant, *n12*, at high multiplicities of infection (43) and that ICP27 is involved in determining the intracellular localization of ICP0 and certain mutant forms of ICP4 in ICP4 mutant virus-infected cells (43, 44). Moreover, ICP27 has inhibitory effects on the nuclear localization of ICP0 and -4 in transient-transfection assays (43). Consistent with these previous studies, we have found that both ICP0 and mutant forms of ICP4 are present to various degrees in the cytoplasm of cells infected with ICP4 *ts* mutant viruses at high multiplicities of infection at the NPT and that ICP0 is also present predominantly in the cytoplasm of cells infected at high multiplicities with ICP4 nonsense and deletion mutant viruses, suggesting that impairment of the normal nuclear localization of ICP0 is a common feature of cells infected with ICP4 mutant viruses. At the inception of these studies, however, it remained unclear

FIG. 5. Localization of ICP0 and ICP27 in Vero or 3-3 cells infected with the ICP4-ICP27 double-mutant virus, *d92*. (Top) ICP27-expressing cells (3-3) were infected at 37°C with 0.5 (A), 5 (B), or 20 (C) PFU of *d92* per cell, and Vero cells were infected at 37°C with 20 PFU of *d92* per cell (D). At 5 h postinfection, cells were processed for IF and stained for ICP0 with mouse anti-ICP0 monoclonal antibody H1083 as the primary antibody. (Bottom) 3-3 cells were infected at 37°C with 0.5 (E), 5 (F), or 20 (G) PFU of *d92* per cell. At 5 h postinfection, cells were processed for IF and double stained for ICP27 and ICP0 as described in Materials and Methods. ICP27-specific staining is shown in panels E, F, and G, and ICP0-specific staining (in the same cells as shown in panel F) is shown in panel H. The proteins examined, the cells used to infect with *d92*, and the multiplicities used in infection are indicated adjacent to each micrograph. MOI, multiplicity of infection.

whether mutant ICP4 polypeptides themselves or the absence of functional ICP4 was responsible for determining the cytoplasmic localization of ICP0.

In an attempt to determine whether mutant ICP4 polypeptides are able to inhibit the nuclear localization of ICP0, cotransfection experiments were performed. In these tests, it was found that a variety of mutant forms of ICP4 failed to displace ICP0 from the nucleus in transient-transfection assays. On the basis of this observation, we concluded that it would be unlikely that during infection, mutant forms of ICP4 specified by viruses containing the same mutations as these plasmids would inhibit the nuclear localization of ICP0.

Extensive studies of a variety of ICP4 mutant viruses, including *ts*, nonsense, and deletion mutants, revealed no correlation between mutant forms of ICP4 and the aberrant localization pattern of ICP0. These observations lead us to hypothesize that *ts* forms of ICP4, like other mutant forms of ICP4, have no inhibitory effect on the nuclear localization of ICP0. In support of this hypothesis, Gelman and Silverstein tested the *tsK* allele of ICP4 and found that this form of ICP4 was localized predominantly to the nucleus and did not inhibit the nuclear localization of ICP0 in transient-transfection assays (16).

This study and previous studies (43, 44) have shown that ICP27 is critical in determining the intracellular localization of ICP0 and certain mutant forms of ICP4 in ICP4 mutant virus-infected cells. Although the mechanism by which ICP27 affects the nuclear localization of ICP0 and ICP4 is unknown, it is reasonable to postulate that protein-protein interactions (direct or indirect) and protein modification are involved, since specific regions of all three proteins required by ICP27 to regulate the intracellular localization of ICP0 and ICP4 have been mapped (43, 44) and since all three proteins are nuclear phosphoproteins (1, 28, 42). In cells infected with 20 PFU (Fig. 1 and 2) but not in cells infected with 0.5 PFU (Fig. 3) of ICP4 mutants per cell, ICP0 and certain mutant forms of ICP4 are localized either predominantly in the cytoplasm or in both the cytoplasm and the nucleus with a greater cytoplasmic component than that of wild-type virus-infected cells. The simplest explanation for this phenomenon is that the cytoplasmic localization of ICP0 and certain mutant forms of ICP4 is due to the saturation of the nucleus with these proteins. This explanation alone seems unlikely, however, because the cytoplasmic staining components of these two proteins were much more prominent than their nuclear components in many cells, and in some

FIG. 6. Levels of expression of ICP27 in infected cells. 3-3 cells were infected with the ICP4-ICP27 double-mutant virus, *d92*, at 0.5, 5, 20, or 100 PFU per cell (lanes 2 to 5, respectively); with the ICP27 mutant, *5dl1.2*, at 5, or 20 PFU per cell (lanes 6 and 7, respectively); or mock infected (lane 1 and 8). As controls, Vero cells were infected at 20 PFU per cell with the wild-type virus, KOS (lane 9); with *5dl1.2* (lane 10); with *d92* (lane 11); with the ICP0 null mutant, 7134 (lane 12); or with the ICP4 mutant, *n12* (lane 13). At 5 h postinfection, the levels of expression of ICP27 were detected by immunoblotting analysis with mouse monoclonal antibody H1113 as described in Materials and Methods. moi, multiplicity of infection.

cells, only very little nuclear ICP0 and ICP4 staining was detected.

With regard to the localization patterns of ICP0 and ICP4 in *tsK*-infected cells, Knipe and Smith (21) reported that the aberrant localization of ICP0 and ICP4 was not observed in Vero cells infected at a multiplicity of infection of 20 PFU per cell or less and that this altered localization was observed only in a small proportion of Vero cells infected at a higher multiplicity, e.g., 40 PFU per cell (20). Our results are inconsistent with those results in that the multiplicity at which the aberrant localization patterns of ICP0 and ICP4 were observed differs, but they are consistent with those results in that the localization patterns of ICP0 and ICP4 were found to be multiplicity dependent. Since the localization patterns of ICP0 and ICP4 varied as a function of multiplicity of infection in both studies, the discrepancy between these two studies likely results from technical differences, such as titers of virus stocks, particle-to-PFU ratios, and incubation temperatures, etc.

ICP0 is recognized as a potent and broad transactivator of a spectrum of eukaryotic promoters. It is able to activate gene expression from many promoters, including a number of heterologous viral and cellular promoters in transient-transfection assays, but it fails to activate E and L gene expression in cells infected with mutant viruses lacking functional ICP4 (11). This long-recognized fact is paradoxical with regard to the regulation of HSV gene expression. Our studies have shown that the localization site of ICP0 is multiplicity dependent in ICP4 mutant virus-infected cells and that high levels of ICP27 resulting from the inability of mutant forms of ICP4 to repress the expression of IE genes are responsible for the cytoplasmic localization of ICP0. Therefore, our studies may provide an explanation for the inability of ICP0 to activate gene expression during high-multiplicity infection with mutants lacking functional ICP4. Since transactivators function in the nucleus, the cytoplasmic localization of ICP0 in ICP4 mutant virus-

infected cells would prevent ICP0 from performing its transactivating function. On the other hand, even though ICP0 localizes exclusively within the nucleus, the expression of E and L genes is more restricted in *d92*-infected cells than in cells infected with either *d120* or *5dl1.2* (36). This observation suggests that the inability of ICP0 to activate the expression of E and L genes in ICP4 mutant virus-infected cells is not due solely to the cytoplasmic localization of ICP0 and that other factors, perhaps including ICP4 and/or ICP27, may also be involved in the control of the transactivating activity of ICP0 or the activities of the promoters of E and L genes in the context of the viral genome. Notably, similar conclusions were reached by Mullen et al. (25a). Tests to determine whether ICP0 expressed in *d92*-infected cells is able to activate gene expression in transfection-superinfection assays should shed light on the function of ICP0.

ICP0, ICP4, and ICP27 are IE proteins that control viral gene expression cooperatively during productive infection. The transregulatory activities of these proteins must be bypassed during the establishment and maintenance of latency, when viral gene expression is repressed. To explore the molecular basis for the functional cooperativity among these three proteins in the regulation of HSV-1 gene expression, we have examined their intracellular localization in this study and previous studies (43, 44). Collectively, our results demonstrate that ICP27 has an inhibitory effect on the nuclear localization of ICP0 and ICP4, indicating that the mechanism of regulation of HSV-1 gene expression may well involve intracellular compartmental constraints. It will be of interest to determine whether intracellular compartmental constraints are involved in the establishment and maintenance of latency.

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