Association of ICP0 but Not ICP27 with Purified Virions of Herpes Simplex Virus Type 1

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Recent studies have shown that ICP4, one of the major immediate-early proteins of herpes simplex virus type 1 is present within the tegument region of the virion (F. Yao and R. J. Courtney, *J. Virol.* 63:3338–3344, 1989). With monoclonal antibodies to two additional immediate-early proteins, ICP0 and ICP27, and Western blot (immunoblot) analysis, ICP0, but not ICP27, was also found to be associated with purified virus particles. In an effort to localize the ICP0 within the virion, purified virions were treated with trypsin in the presence and absence of detergent. The data suggest that ICP0 is located within the tegument region of the virion and is not localized in the envelope or within the nucleocapsid. The number of molecules of ICP0 per virion was estimated to be approximately 150.

The immediate-early or alpha genes of herpes simplex virus type 1 (HSV-1) are the first viral genes expressed during the infectious cycle. These genes are expressed in the absence of prior viral protein synthesis and specify five proteins designated infected-cell polypeptides (ICP): ICP0, ICP4, ICP22, ICP27, and ICP47 (8, 27, 38). To date, there is no evidence indicating that ICP22 or ICP47 affects viral gene expression in transient expression assays (13, 17). In contrast, ICP0, ICP4, and ICP27 are involved in regulating immediate-early, early, or late gene expression (12, 15, 18, 22, 35, 36, 46, 49). Studies of temperature-sensitive, non-sense, and deletion mutant viruses in these five immediate-early genes have indicated that ICP4 and ICP27 represent essential gene products in HSV-1-infected cell cultures (12, 15, 41, 44). The immediate-early protein, ICP4 or Vmw175 (apparent molecular weight of 175,000), is a major regulatory protein which modulates viral gene expression during virus replication (12, 14, 15, 41). ICP27, on the other hand, plays an essential role in the regulation of early and late gene expression and is required for virus replication (35, 44).

Although ICP0 is not essential with regard to virus replication (45, 48), the results from analysis of mutant viruses with deletions and insertions in the gene encoding ICP0 suggest that the existence of a functional ICP0 provides a selective advantage on virus growth in tissue culture (5, 20, 45, 48). ICP0 is predominantly located in the nuclei of infected cells and is a phosphorylated protein with an apparent molecular weight of 110,000 (28, 38, 51). Studies have demonstrated that ICP0 may function as a regulatory protein to activate the expression of all three classes of viral genes and a number of heterologous promoters either by itself or in a synergistic fashion with ICP4 (5, 17, 19, 22, 34, 37, 42). The amino acid sequence analysis of ICP0 (39) shows that it contains zinc finger structural motifs characteristic of a class of eukaryotic transcription factors, suggest-

ing that the mechanism of transcriptional activation probably involves the binding of ICP0 to DNA. Other studies have suggested that ICP0 plays a major role in the reactivation of latent herpes simplex virus genomes in both in vitro (43) and in vivo (31) latency systems. Recent experiments have indicated that ICP0 alone can reactivate HSV-2 from latency in an in vitro system (25).

We have reported that one of the immediate-early proteins, ICP4, a major transcriptional regulatory protein of HSV-1, is associated with purified HSV-1 virions (52). Studies have shown that approximately 100 to 200 molecules of ICP4 are located within the tegument region of virus particles. It was of interest to determine whether other immediate-early proteins, particularly ICP0 and ICP27, are also located within the virion. In this report, we present data that demonstrate that detectable amounts of ICP0 are also present within the tegument region of purified virions. In contrast, no ICP27 was detected in purified virus particles. Since significant amounts of ICP0, ICP4, and ICP27 are all localized within the nucleus, these findings provide additional evidence that the association of ICP4 and ICP0 with HSV-1 virions represents a specific and not a random event.

MATERIALS AND METHODS

Cell culture and virus. African green monkey kidney (Vero) cells were grown in Eagle's medium supplemented with 5% fetal bovine serum and 0.075% sodium bicarbonate. The KOS strain of HSV-1 was grown in human embryonic lung fibroblasts (MRC-5), and all virus titrations were conducted with Vero cell monolayers (2).

Infection of cells and purification of virions. Monolayers of Vero cells cultured in roller bottles (850 cm²) were infected with HSV-1 at a multiplicity of 3 PFU per cell. After 1 h of adsorption at 37°C, maintenance medium containing 2% serum was added. All incubations were carried out at 37°C. Infected cells were labeled with [³⁵S]methionine (5 µCi/ml) from 4 to 26 h after infection. At 26 h postinfection, virions were purified by using methods previously described (9, 51). The infectivity of purified viruses was measured, and the physical-particle-to-PFU ratio of purified virions was approximately 100. These results indicated that the virus purification procedure did not significantly reduce the virion

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infectivity. As an alternative approach for virus purification, extracellular virions were isolated by a method essentially as described by Spear and Roizman (47). After removal of cell debris by low-speed centrifugation of medium harvested from HSV-1-infected Vero cells at 30 to 36 h postinfection, virions were pelleted from the supernatant and suspended in TNE buffer (10 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA). The virus suspension was then layered onto a 5 to 25% (wt/vol) continuous dextran 10 gradient and centrifuged for 1 h at 20,000 rpm in an SW28 rotor. Following centrifugation, a diffuse light-scattering virus band located near the middle of the tube was collected by puncturing the side of the tube with a needle and syringe. The virus from the dextran gradient was treated with 0.5 M urea, sonicated for 5 s, and was mixed with sucrose to a final concentration of 50% (wt/wt). A discontinuous gradient was prepared by the sequential addition of 40, 30, and 20% (wt/wt) sucrose to the virus suspension followed by centrifugation at 25,000 rpm for 18 h in an SW28 rotor. After centrifugation, a virus band at the 40 to 50% interface was collected and diluted in phosphate-buffered saline (PBS). This virus suspension was then layered onto a solution of 10% sucrose in 2 M urea and PBS and subsequently centrifuged at 25,000 rpm for 2 h in an SW28 rotor. The pelleted virus was suspended in PBS and stored at -80°C .

Detergent treatment of purified virions. Purified virions were treated with 0.5% Triton X-100 and 0.5% deoxycholate for 15 min at 37°C . The reaction mixture was separated into two fractions, designated the supernatant and pelleted virus particles, by centrifugation at $70,000 \times g$ for 1 h.

Trypsin treatment of purified virions. Purified HSV-1 virions were treated with 50 μg of trypsin per ml in either the absence or presence of 1% Triton X-100 for 10 min at 37°C . The proteolysis reaction was terminated by the addition of 0.5 mg of soybean-trypsin inhibitor per ml and 0.4 mM phenylmethylsulfonyl fluoride. Proteins were precipitated with acetone prior to their analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Isolation of intranuclear virus capsids. Type A and B capsids were isolated from HSV-1-infected HEP-2 cells at 22 h postinfection by methods described previously (24, 52).

SDS-PAGE and immunoblotting. Details of the methods used for SDS-PAGE have been previously described (40). All slab gels were 7% acrylamide and were either dried onto filter paper and exposed to X-Omat film or transferred onto nitrocellulose paper and analyzed by Western blot (immunoblot). Immunoblotting with hyperimmune monospecific rabbit antisera to either glycoproteins gB and gC (16), gH, tegument protein VP16, or ICP4 (11) was performed essentially as previously described (9). The anti-gH rabbit serum was kindly provided by Gary Cohen and Roselyn Eisenberg, University of Pennsylvania. The polyclonal rabbit serum specific for VP16 was generously provided by Steven McKnight, Carnegie Institution of Washington, Baltimore, Md. Procedures for Western blot analysis with mouse monoclonal antibodies specific for ICP0 or ICP27 (generously provided by Lenore Pereira, University of California, San Francisco, Calif.) were as follows. The proteins resolved by SDS-PAGE were transferred to nitrocellulose paper, which was then treated with bovine serum albumin as previously described (9). After 1 h of incubation at room temperature, the paper was rinsed with water. Monoclonal antibodies specific for ICP0 and ICP27 were diluted 1:1,000 and 1:200, respectively, in $1\times$ NETG buffer ($1\times$ NETG is 150 mM NaCl, 5 mM EDTA, 50 mM Tris hydrochloride [pH 7.4], 0.25% gelatin) containing 0.05% Nonidet P-40 and incubated

FIG. 1. ICP4 and ICP0, but not ICP27, are associated with purified virions. HEP-2 cells were infected with HSV-1 (KOS), and extracellular virions were purified by using the procedure described in Materials and Methods. Proteins of purified virions (lanes 1) or infected-cell extracts (lanes 2) were resolved by SDS-PAGE, transferred to nitrocellulose paper, and immunoblotted with either polyclonal, monospecific anti-ICP4 serum (A) or monoclonal antibodies to ICP0 (B) and ICP27 (C).

for 90 min with the blot. The excess antibody was removed by washing the blots with 10 20-ml volumes of $1\times$ NETG buffer containing 0.1% SDS and 0.5% Triton X-100. The nitrocellulose paper was then incubated for 1 h with rabbit anti-mouse immunoglobulin G with the same dilution as that used for the anti-ICP0 or anti-ICP27 monoclonal antibodies. After the blots were washed as described above, the reaction of the nitrocellulose paper with ^{125}I -protein A for 90 min was followed by a repetition of the washing steps and a final rinse in water. The blots were air dried and exposed on Kodak X-Omat film.

RESULTS

Association of ICP0, but not ICP27, with purified virions.

Proteins from virions purified according to a procedure previously described (52) were resolved by SDS-PAGE, transferred to nitrocellulose paper, and immunoblotted with antibodies specific for either ICP4, ICP0, or ICP27. HSV-1-infected cell lysates were also included as positive control samples. As shown in Fig. 1, ICP4, ICP0, and ICP27 all were present in infected-cell extracts (lanes 2 of panels A, B, and C, respectively). The detection of reduced amounts of ICP0 in cell lysates was highly reproducible and may reflect the instability of this protein within the infected cell (51). As reported previously (4), ICP4 was detected in association with purified virions (Fig. 1A, lane 1). In addition, ICP0, but not ICP27, was associated with purified virions (Fig. 1B and C, lanes 1). Since ICP4, ICP0, and ICP27 all are phosphorylated proteins and predominantly localized within the nuclei of virus-infected cells (4, 28, 29, 38, 51), the lack of detection of ICP27 in purified virions may further reflect the specific nature of incorporation of ICP4 and ICP0 into virus particles.

To confirm further the association of ICP4 and ICP0 with purified virions, an additional virus purification protocol was used. ^{14}C -amino acid-labeled virions obtained from the extracellular medium were purified by the procedures described above (52) (Fig. 2, lanes 2) and by the procedures of Spear and Roizman (47) (Fig. 2, lanes 1). The proteins of both purified virion preparations were then resolved by SDS-PAGE, and the gels were either directly analyzed by autoradiography of the ^{14}C -amino acid-labeled proteins (Fig.

FIG. 2. Association of ICP0 and ICP4 with virions purified by two different procedures. ^{14}C -amino acid-labeled extracellular virions were purified according to the procedures described by Spear and Roizman (47) (lanes 1) and our laboratory (52) (lanes 2). Virion proteins were analyzed by SDS-PAGE and either directly visualized by an autoradiography of the ^{14}C -amino acid-labeled proteins (A) or transferred onto nitrocellulose and analyzed by Western blot with anti-ICP0 (B) and anti-ICP4 (C) monospecific antibodies.

2A) or transferred onto nitrocellulose and immunoblotted with anti-ICP0 (Fig. 2B) and anti-ICP4 (Fig. 2C) antibodies. As shown in Fig. 2A, an approximately equal number of viral particles were present in both samples (note intensity of the VP5 band), and the two different virion purification procedures yielded essentially the same ^{14}C -amino acid-labeled virion protein profiles. The results from immunoblotting analyses (Fig. 2B and C) confirmed the possible association of ICP0 and ICP4 with HSV-1 virions regardless of the purification procedures employed.

In an attempt to demonstrate further that ICP0 was indeed associated with purified virus particles, virions were centrifuged in a sucrose gradient and the various fractions were monitored by SDS-PAGE. The objective of the experiment was to determine whether the location of ICP0 in the sucrose gradient coincided with that of the major structural proteins of the virion. Extracellular virions were obtained from HSV-1-infected Vero cells labeled with [^{35}S]methionine from 4 to 26 h postinfection. The virions were layered onto a 20 to 60% linear sucrose gradient and centrifuged as previously described (52). Fractions were collected from the gradient and assayed by SDS-PAGE followed by autoradiography of the [^{35}S]methionine-labeled proteins or by immunoblotting with the anti-ICP0 monoclonal antibody. Significant amounts of viral structural proteins, such as the major capsid protein, VP5, were detected in fractions 7, 8 and 9 (Fig. 3A). The results presented in Fig. 3B indicate that a protein reactive with the anti-ICP0 antibody was mainly detectable in fractions 8 and 9. In an attempt to further verify that the fractions containing anti-ICP0 reactivity represent the virion peak, the infectivity of each fraction was determined by analysis for PFU. The data shown in Fig. 4 demonstrate that fractions 8 and 9 contained the highest titers of infectious units and this finding provides further support that ICP0 is associated with purified virions.

Effect of detergent and trypsin treatment on the association of ICP0 with purified virions. The data presented above indicate that ICP0, like ICP4, was associated with virus particles. Experiments were designed to determine whether

FIG. 3. Protein reactive with ICP0 monoclonal antibody cofractionates with virus particles. Extracellular virions labeled with [^{35}S]methionine were resolved from nonvirion-associated proteins on a 20 to 60% linear sucrose gradient by centrifugation for 20 h at 20,000 rpm in an SW41 rotor. Following centrifugation, individual fractions were collected and analyzed by SDS-PAGE and immunoblotted with an anti-ICP0 monoclonal antibody. (A) Autoradiogram of [^{35}S]methionine-labeled protein profiles of fractions obtained from the sucrose gradient; (B) autoradiogram of the anti-ICP0 immunoblot of the same samples analyzed in panel A. The fraction numbers are given above the lanes. B and T refer to the bottom and top of the centrifuge tube.

ICP0 is located within the envelope, tegument, or nucleocapsid of the virion. First, purified virions were treated with 0.5% Triton X-100 and 0.5% deoxycholate and then subjected to high-speed centrifugation. The supernatant and pelleted virus particles were analyzed by SDS-PAGE (Fig. 5) and immunoblotted with either anti-ICP4-, anti-ICP0-, or anti-gC-specific antibody (Fig. 5A, B, and C, respectively). Lane 1 of each panel represents the untreated virion control, and lanes 2 and 3 contain the supernatant and pelleted virus particles, respectively. The data in panel C show that over 90% of the viral envelope glycoprotein, gC, was solubilized from virus particles after detergent treatment. However, neither ICP4 nor ICP0 (panels A and B, respectively) was detected in the supernatant fraction, indicating that these two proteins are not released from the virion by detergent treatment.

In an effort to define the location of ICP0 in the virus particle, purified virions were treated with trypsin in the presence or absence of detergent. Since ICP0 was not released by detergent treatment, these data suggest that ICP0

FIG. 4. Anti-ICP0 reactivity cosediments with fractions containing HSV-1 infectious particles. The infectivity titer of each fraction shown in Fig. 3 was determined by analysis for PFU on Vero cell monolayers (2). B and T refer to the bottom and top of the centrifuge tube.

was located internal to the envelope. Therefore, when one treats the virions with trypsin in the absence of detergent, the envelope should prevent the trypsin from digesting the ICP0 molecules. Purified virions were treated with trypsin in the presence and absence of detergent as described in Materials and Methods. The samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with either anti-ICP4-, anti-ICP0-, or anti-gB-specific antibody. The results presented in Fig. 6 show that as expected, glycoprotein gB (Fig. 6C) was sensitive to trypsin treatment in both the presence (lane 2) and absence (lane 3) of detergent. The data presented in Fig. 6A and B show that ICP4 and ICP0 were sensitive to trypsin treatment only when detergent was present (lane 2). In contrast, trypsin treatment alone did not affect the ability to detect ICP0 in the purified virions (lane 3).

These data indicate that ICP0, like ICP4, was located within the virion.

To further localize ICP0 within the virus particle, additional studies were conducted to determine whether ICP0 was associated with viral nucleocapsids isolated from the nuclear fraction of HSV-1-infected cells. By using procedures described previously (24, 52), the viral nucleocapsids were purified and analyzed for the presence of ICP0 (Fig. 7). Panel A shows [³⁵S]methionine-labeled protein profiles of purified virions as well as those of type A and B capsids. The type B capsid was characterized by the presence of a protein designated VP22a (24). Panels B, C, and D represent Western blot analyses of the resolved proteins shown in panel A with anti-ICP0, anti-ICP4, and anti-VP16 antibodies, respectively. As indicated, ICP0, like VP16 and ICP4, was not associated with the type A and B capsids. Taken together, these results suggest that ICP0 is likely associated with the tegument region of the virion.

Quantitation of the amount of ICP0 within the virion. HSV-1-infected Vero cells were labeled with a ¹⁴C-amino acid mixture (1 μ Ci/ml) at 2 h postinfection, and extracellular virions were harvested and purified after 24 h of infection as described in Materials and Methods. However, it was difficult to distinguish ICP0 from glycoprotein gH according to their migrations by SDS-PAGE, and a definitive estimate of the number of molecules of ICP0 per virion from the autoradiogram of the ¹⁴C-amino acid-labeled virion protein profile was not possible. Since glycoproteins gB, gC, and gH are localized in the envelope region of the virion and ICP0 is within the tegument, the glycoproteins versus a tegument protein will have different sensitivities to trypsin treatment. Therefore, the number of ICP0 molecules per virion was estimated after the migration of the glycoproteins by SDS-PAGE was altered by trypsin treatment. Purified virions were treated with trypsin in the absence of detergent as described in Materials and Methods. After trypsin treatment, the samples were resolved by SDS-PAGE, and either the gels were directly visualized by autoradiography of ¹⁴C-amino acid-labeled proteins (Fig. 8A) or the resolved proteins were transferred onto nitrocellulose paper and immunoblotted with either anti-ICP0, anti-gC, or anti-gH antibodies. As shown in Fig. 8D, glycoprotein gH was

FIG. 5. Determination of the fate of virion-associated ICP0 following detergent treatment of purified virions. Virions were reacted with 0.5% Triton X-100 and 0.5% deoxycholate for 15 min at 37°C and then centrifuged at 70,000 $\times g$ for 1 h to separate the reaction mixtures into two fractions, the supernatant (lanes 2) and the pelleted virus particles (lanes 3). The samples were assayed by SDS-PAGE and immunoblotted with either anti-ICP4 (A), anti-ICP0 (B), or anti-gC (C) antibodies. Lane 1 in each panel contains mock-treated virions.

FIG. 6. Protection of virion-associated ICP0 from trypsin treatment by the virus envelope. Purified HSV-1 virions were reacted with trypsin (50 μ g/ml) in either the absence (lane 3) or presence of 1% Triton-X 100 (lane 2) for 10 min at 37°C, as described in Materials and Methods. The samples were analyzed by SDS-PAGE and immunoblotted with anti-ICP4 (A), anti-ICP0 (B), or anti-gB (C) antibodies. Lane 1 in each panel contains mock-treated purified virions.

completely sensitive to trypsin treatment. Glycoprotein gC was less sensitive to trypsin and always yielded detectable trypsin digestion products. Again, the data in Fig. 8B demonstrate that ICP0 was protected from trypsin digestion by the viral envelope. When the identical sample was immunoblotted with anti-gB serum, the same result as shown in Fig. 6C was obtained (data not shown). Therefore, we conclude that the band marked by an asterisk in Fig. 8A, lane 2, represents ICP0. The amount of ICP0 present in virions was quantitated by densitometric scanning of lanes 1 and 2 by using a laser scanning densitometer. By the method of Heine et al. (26), it was estimated that there are approximately 150 molecules of ICP0 per virion.

DISCUSSION

Recent studies in our laboratory have shown that ICP4, the major regulatory protein of HSV-1, is associated with purified virions (52). The data presented in this report

demonstrate that, in addition to ICP4, the transacting regulatory protein ICP0 of HSV-1 was also detected in purified virions. Furthermore, the results indicate that the virus-associated ICP0 is located within the tegument region of the virus particles and each HSV-1 virion contains approximately 150 molecules of ICP0. We suggest that the incorporation of ICP4 and ICP0 into the virion represents a highly specific event on the basis of the following observations. First, although significant amounts of all three immediate-early proteins (ICP4, ICP0, and ICP27) are localized within the nucleus, only ICP4 and ICP0 were detectable within the purified virions. Second, the nuclear-associated 65-kDa DNA binding protein was not detected in purified virions (52). Third, studies with certain nonsense mutants of ICP0 (kindly provided by Priscilla Schaffer and Weizhong Cai, Harvard Medical School) have shown that even though truncated forms of ICP0 specified by certain mutants accumulate within the nuclear fraction, their incorporation into

FIG. 7. Detection of ICP0 with type A and B capsids purified from the nuclear fraction of HSV-1-infected cells. Viral capsids were isolated from the nuclear fraction of virus-infected cells by using methods described by Gibson and Roizman (24). (A) [35 S]methionine-labeled protein profiles of purified virions (v) as well as purified type A (C_A) and B (C_B) capsids; (B, C, and D) Western blot analyses of the identical samples shown in panel A with anti-ICP0, anti-ICP4, and anti-VP16 antibodies, respectively. The band indicated by an asterisk in panel A represents the type B virus capsid-specific protein, VP22a (24).

FIG. 8. Estimation of the number of ICP0 molecules per virion. ^{14}C -amino acid-labeled virions were treated with $50\ \mu\text{g}$ of trypsin per ml (lanes 2) in the absence of detergent for 10 min at 37°C . The trypsin was inactivated by the addition of trypsin inhibitors, and the proteins were precipitated with acetone. The ^{14}C -amino acid-labeled proteins of purified virions were resolved by SDS-PAGE and either directly visualized by autoradiography (A) or transferred onto nitrocellulose paper and blotted with anti-ICP0 (B), anti-gC (C), or anti-gH (D) antibodies. Lane 1 in each panel represents mock-treated virions. The band marked by an asterisk in lane 2 of panel A represents ICP0.

the tegument region of the virus particle is defective (unpublished results), whereas the ICP0 protein expressed by another ICP0 mutant was specifically incorporated into HSV-1 virus particles (data not shown).

Among the five immediate-early proteins of HSV-1, only ICP0, ICP4, and ICP27 are reported to be involved in regulating HSV-1 gene expression in transient expression assays (5, 12, 15, 18, 22, 35, 36, 46, 49). In addition to its ability to transactivate HSV-1 early and late genes, ICP4 can either repress or induce its own synthesis and possibly that of the other immediate-early genes in a concentration-dependent manner (14, 22, 36). ICP0, on the other hand, can stimulate all three kinetic classes of viral gene expression and, in some instances, may function in a synergistic manner with ICP4 (5, 17, 19, 22, 34, 37, 42). In fact, it has been suggested that ICP0 may physically associate with ICP4 (19). In contrast, there is no evidence to date suggesting that ICP27 has a direct effect on up-regulating the expression of the HSV-1 immediate-early genes.

It has been shown that transcription of the five immediate-early genes requires no prior viral protein or DNA synthesis (8). Each of the five immediate-early genes contains a consensus sequence (TAATGARAT) which is located 5' to the mRNA start site (10, 33). Studies using either stably transformed cells lines or transient expression assays have indicated that this consensus sequence is intimately involved in transcriptional regulation mediated by a 65-kDa structural protein located within the tegument region of the infecting virion (1, 3, 6, 21, 30, 37). This viral structural protein has been variably designated VP16, Vmw65, or α -trans-inducing factor (1, 6, 50). Notably, VP16 alone is incapable of binding to viral DNA, and its transactivation function is mediated by interacting with the host cell ubiquitous octamer-binding protein, OTF-1 (23, 50).

The finding that in addition to VP16, ICP0 and ICP4 are present in the tegument region of the purified HSV-1 virions may provide insight into the additional role that these two HSV-1 immediate-early regulatory proteins play with respect to modulating viral gene expression during the initial stages of productive infection. On the basis of the known

regulatory functions of ICP0 and ICP4, it is possible that the virion-associated ICP0 and ICP4 act alone or synergistically with VP16 to activate expression of immediate-early gene at the initial stage of viral productive infection. This hypothesis is strongly supported by the following observations.

A recent report describes the construction of a virus containing a mutation in the transactivating functions of VP16 (7). The viral plating efficiency of this mutant virus at low multiplicities of infection was significantly reduced, and interestingly, this inefficient plaque formation was overcome by transfection of cells with an ICP0-expressing plasmid prior to viral infection. Importantly, in the absence of de novo protein synthesis, the production of ICP0 and ICP27 mRNAs in mutant virus-infected cells was decreased up to fivefold compared with that in wild-type virus-infected cells, whereas the expression of the ICP4 gene was unaffected. When combined with earlier findings (1) that there exists a virion-associated factor(s) capable of inducing the expression of the resident ICP4 promoter-thymidine kinase gene chimera, it seems not surprising for one to speculate that virion-associated ICP0 and/or ICP4 may represent virion-associated factors that participate, at least partially, to enhance the expression of ICP4 and possibly other immediate-early genes at the very early stages of viral replication. The major function of VP16 at the initial stages of viral infection might be to induce the expression of ICP0 and ICP27. Although there is ample evidence indicating that ICP0-expressing cell lines could significantly increase the plating efficiency of ICP0 mutant virus, the physical-particle-to-PFU ratio of mutant virus remains approximately 10-fold higher than that of wild-type virus. Notably, the level of expression of resident ICP0 in mutant virus-infected ICP0-expressing cells is much lower than that of wild-type virus-infected cells (43); this lower level of expression might be the main reason why ICP0-expressing cells are not able to fully compensate the mutant virus growth. Whether the low level of expression of ICP0 in mutant virus-infected ICP0-expressing cells is due to the absence of functional virion-associated ICP0 to up-regulate its expression at the initial stages of viral infection or whether the resident ICP0 promoter is less active than that in the context of the viral genome represents an interesting and important question to be answered.

The results presented within this study suggest that approximately 100 to 200 molecules of ICP0 and ICP4 are associated with each virion. Although this is significantly less than the number of VP16 molecules present in virions, it remains a significant number of molecules present per virion genome. If the virion-associated ICP4 and ICP0 are functional, one might expect to see expression of early proteins prior to the de novo synthesis of immediate-early proteins. Currently, all reports suggest a strict requirement for the de novo synthesis of immediate-early proteins before the expression of early genes. On the basis of the data currently available, this requirement would suggest that either the number of molecules associated with the virion is too few to activate early gene expression or the virion-associated ICP0 and ICP4 are nonfunctional with respect to the viral early promoters during the very early stages of viral replication. The latter possibility might be supported by the facts that (i) only the low-molecular-weight species of ICP4 is present within the HSV-1 virion and (ii) the effect of ICP0 on early and late gene expression is dependent on the existence of functional ICP4 in the context of viral infection.

As mentioned above, the transactivating function of VP16 is mediated by both the *cis*-acting sequence TAATGARAT and the cellular ubiquitous octamer-binding protein, OTF-1.

It is also possible that the virion-associated ICP0 and ICP4 may function as a back-up transactivator to up-regulate the synthesis of immediate-early genes at the initial stages of productive infection in cells that the transactivation activity of VP16 on immediate-early gene promoters is ineffective or eliminated by the presence of certain cell-type-specific repressor(s). For example, studies by Lillycrop et al. (32) indicated that there exists a neuron- and lymphocyte-specific octamer-binding factor, oct2, that can specifically bind to the TAATGARAT sequence and results in reducing expression of HSV-1 immediate-early genes. Earlier studies by Gerster and Roeder (23) have demonstrated that oct2 is incapable of interacting with VP16.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA42460 and AI29026.

We are grateful to Jacqueline Fetherston and Quan Zhu for helpful discussions and critically reading the manuscript, Sally Weldon for excellent technical assistance, and Carol Ann Ardoin for preparation of the manuscript.

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