Herpes Simplex Virus Type 1 ICP27 Deletion Mutants Exhibit Altered Patterns of Transcription and Are DNA Deficient

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Received 23 June 1988/Accepted 9 September 1988

Infected cell polypeptide 27 (ICP27, α27, IE63) is the 63-kilodalton product of an immediate-early gene of herpes simplex virus. Functional analysis of temperature-sensitive mutants in herpes simplex virus type 1 ICP27 demonstrated that this protein plays an essential role in virus replication (W. R. Sacks, C. C. Greene, D. P. Aschman, and P. A. Schaffer, J. Virol. 55:796–805, 1985). Because the temperature-sensitive forms of ICP27 induced by the mutants affected gene expression to differing degrees, these mutants were not suitable for establishing the ICP27 null phenotype. For this purpose we generated deletion mutants in ICP27—Δd11.2 and Δd11.2—lacking the transcriptional start site as well as portions of the promoter and coding sequences of the gene. These mutants failed to specify ICP27-specific transcripts and proteins and were replication incompetent. The mutants induced the synthesis of greatly reduced levels of viral DNA (18% of wild-type levels) and were characterized by the overexpression of early proteins, reduced levels of γ1 proteins, and the absence of detectable γ2 proteins. The alterations in viral protein synthesis appeared to occur at the level of transcription. The phenotypic properties of the mutants were consistent with the results of transient expression assays demonstrating that ICP27 acts to down-regulate transcription of early genes and to further up-regulate transcription of late genes whose expression is induced by ICP0 and ICP4. Because ICP27 is not thought to be directly involved in viral DNA synthesis, it is likely that the reduced levels of viral DNA characteristic of deletion mutant-infected cells is a consequence of aberrant regulation of certain early genes whose products are involved in viral DNA synthesis and late genes whose products are required to stabilize viral DNA once synthesized. Taken together, these findings suggest an essential role for ICP27 in the modulation of early and late gene expression at the transcriptional level.

The herpes simplex virus (HSV) genome encodes approximately 80 viral proteins that are expressed sequentially in three major kinetic classes: immediate early (α), early (β), and late (γ) (6, 21). The mechanisms underlying the sequential and coordinate expression of HSV genes is not yet clear; however, available evidence has implicated the immediate-early proteins as the primary mediators of viral gene expression (8, 12, 22, 37, 41).

Briefly, the program of HSV gene expression can be summarized as follows. Upon infection of susceptible cells, the five immediate-early genes are transcriptionally activated through the agency of a 63-kilodalton protein present in incoming virus particles (2, 5) to yield infected cell polypeptides ICP0, 4, 22, 27, and 47 (35). Transcription of these five genes requires no prior viral protein synthesis (6). Transcription of the second class of viral genes, the early genes, is dependent upon the activities of the immediate-early proteins (12, 22, 37). Synthesis of early proteins—many of which are involved in viral DNA synthesis—signals the onset of viral DNA synthesis, which in turn is required for maximum expression of late genes (20). Late genes specify virion structural proteins and have been divided into two groups, γ1 and γ2. Transcription of γ1 genes from input genomes occurs at low levels in the absence of viral DNA synthesis but is maximal when progeny viral DNA is synthesized (20, 33). By contrast, the accumulation of γ2 transcripts is stringently dependent upon viral DNA synthesis (20, 33). In addition to the requirement for viral DNA synthesis, maximum expression of both γ1 and γ2 genes requires functional immediate-early proteins (20, 22, 33).

With regard to the specific roles of the five immediate-early proteins in the regulation of HSV gene expression, neither ICP22 nor ICP47 has been shown to affect gene expression alone or in the presence of other immediate-early proteins in transient expression assays (10, 13). Notably, the genes for both ICP22 and ICP47 can be deleted from the virus with no adverse affect on viral growth in most cell types (29, 36). Hence neither protein is required for productive infection in vitro.

By contrast, ICP0, 4, and 27 have been implicated in the activation and/or repression of genes in all three kinetic classes. In transient expression assays, ICP0 has been shown to be a potent transactivator of all three classes of viral genes (13, 30, 39) and to act synergistically with ICP4 in the activation of a variety of viral promoters (10, 14, 33). Despite the strength of ICP0 as a transactivator, however, deletion mutants that lack ICP0 are replication competent and exhibit no clear phenotypic alterations when compared with the wild-type virus at high multiplicities of infection (41, 46). They are growth impaired at low multiplicities, however, and induce the synthesis of lower levels of selected early proteins, viral DNA, and most late proteins than does wild-type virus (W. Sacks and P. Schaffer, unpublished observations). Although ICP0, like ICP22 and ICP47, is not essential for productive infection, it has been implicated in the reactivation of HSV from latent infection (D. Leib, D. Coen, C. Bogard, K. Hicks, D. Yeager, D. Knipe, K. Tyler, and P. Schaffer, J. Virol., in press).

Temperature-sensitive mutants and mutants with deletions in ICP4 are not viable under nonpermissive conditions, demonstrating an essential role for this protein in virus replication (8, 12, 38). Specifically, these mutants fail to

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induce the synthesis of early and late proteins and viral DNA and to down-regulate immediate-early protein synthesis (8, 12, 38). Transient expression assays have confirmed the ability of ICP4 to transactivate early and late genes (10, 13, 14, 33). ICP4-mediated regulation of viral gene expression occurs at the level of transcription and may involve the binding of ICP4—either directly or indirectly—to specific target sequences in the viral genome (11, 31).

ICP27 is a 63-kilodalton immediate-early phosphoprotein that localizes to the nucleus of infected cells (23, 32, 48). This protein is encoded by a 2-kilobase (kb) transcript that begins 270 base pairs to the right of the BamHI site at coordinate 0.745 and terminates 150 base pairs to the left of the HindIII site at coordinate 0.761 (Fig. 1). Although the specific function of ICP27 in the HSV replicative cycle is not clear, it is likely that this function is of central importance since an ICP27 homolog is also present in the genome of Epstein-Barr virus (7). Like ts mutants in ICP4, ts mutants in ICP27 are not viable at the nonpermissive temperature, demonstrating an essential role for this protein in productive infection (41). Specifically, four ts mutants in ICP27 induced the synthesis of substantial amounts of early proteins and viral DNA but failed to induce the synthesis of late proteins of the γ2 class at 39°C. Thus it was concluded that ICP27 is not absolutely required for the synthesis of viral DNA but is required for γ2 protein synthesis. Despite the qualitative similarities in the phenotypes of the ts mutants, they differed markedly in the quantities of viral DNA and late polypeptides of the γ1 class they induced at the nonpermissive temperature. Notably, these differences could not be ascribed to varied levels of leakiness, since further elevation in temperature had no effect on the amounts of γ1 polypeptides synthesized. For these reasons, we favor the idea that the different mutational lesions in ICP27 produced mutant polypeptides with differing abilities to facilitate the synthesis of viral DNA and γ1 proteins at the nonpermissive temperature.

Although early studies of the role of ICP27 in viral gene expression with transient expression assays revealed no significant trans-regulating activity for the protein (8, 13), more recent studies indicate that ICP27 can both enhance (14) and repress (40: K. Leary, R. Sekulovich, and R. Sandri-Goldin, unpublished observations) expression of viral genes driven by ICP0 and ICP4. Moreover, Rice and Knipe have recently reported that ICP27 alone can stimulate chloramphenicol acetyltransferase expression under control of the glycoprotein B promoter (40).

Thus, the results of studies with ts mutants and transient expression assays implicate ICP27 as well as ICP0 and ICP4 in the regulation of HSV gene expression. The various abilities of ts forms of ICP27 to affect the synthesis of viral DNA and γ1 proteins, the fact that transient expression assays are not by definition conducted in the context of the viral genome, and the associated inability to directly correlate the results of transient assays with levels of transcription during virus replication are factors that argue against the use of either ts mutants or transient assays for definitive determination of the role of ICP27 in mediating viral gene expression. As a first step in addressing this question, we constructed deletion mutants in ICP27 which lack promoter sequences (26), the transcriptional start site, and sequences encoding 262 amino acids of the amino terminus of ICP27. These mutants were used (i) to determine the phenotypes of mutants lacking the ICP27 protein altogether, (ii) to compare this phenotype with that of ts mutants that produce full-length but temperature-sensitive forms of the polypeptide, and (iii) to ascertain the nature of the block in gene expression characteristic of ICP27 null mutants.

**MATERIALS AND METHODS**

**Cells and viruses.** African green monkey kidney (Vero) cells, ICP4-expressing U-47 cells (34), and ICP27-expressing 3-3 cells were propagated as previously described (41).
HSV type 1 (HSV-1) strain KOS was used as the wild-type virus, and the temperature-sensitive and deletion mutants derived from KOS were propagated and assayed as described previously (43). tsY46 is a temperature-sensitive strain with a mutation in the gene for ICP27 (41), and d21 is a strain with a deletion mutation in the gene for ICP8 (34).

**Plasmids.** The genome locations of viral DNA sequences in plasmids used in this study are shown in Fig. 1. pSG28, containing the EcoRI EK fragment (coordinates 0.724 to 0.865) in pBR325, was kindly provided by R. Sandri-Goldin (University of California, Irvine) (17). pKH-X-BH and pKEB-P123 were generously provided by S. Person (Pennsylvania State University, University Park) and propagated in Escherichia coli RDP145 as previously described (4). p123ABS was derived by removal of the 5.1-kb Nrr1 fragment from pKEB-P123 and insertion of this fragment into the single EcoRI site of pSV2neoRI by using EcoRI linkers. The resulting plasmid was cleaved with BamHI and SalI to introduce a 1.2-kb deletion in the left-hand half of the 5.1-kb fragment. The plasmid was religated in the presence of BglII linkers, creating a new BglII site at the site of the deletion. Bacteria were transformed with the deleted plasmid (p123ABS), and colonies were screened for the presence of the 1.2-kb deletion and the new BglII site. All enzymes and linkers were obtained from New England BioLabs, Inc. (Beverly, Mass.) and used according the manufacturer's specifications. Preparative quantities of plasmid DNA were obtained by the method of Birnboim and Doly (3) and further purified by banding in CsCl.

**Transformation.** Cell lines containing the gene for HSV-1 ICP27 were generated by transformation of Vero cells with a derivative of pKHX-BH (Fig. 1), designated pBHneo. In addition to the 2.4-kb BamHI-to-HpaI fragment, pBHneo contains the neomycin resistance gene from pSV2neo. A 10-μg sample of pBHneo DNA was precipitated in the presence of 30 μg of salmon testis DNA (Sigma Chemical Co., St. Louis, Mo.) in a total volume of 2 ml and used to transfect Vero cells by the method of DeLuca et al. (8). G418-resistant colonies containing the gene for ICP27 were identified by assessing their ability to host the ICP27 mutant, tsY46, at 39°C and were characterized with regard to the integrity and copy number of resident ICP27 genes by Southern blot analysis (8, 45).

**Southern blot analysis.** Restriction enzyme-cleaved DNAs separated by agarose gel electrophoresis were transferred to nitrocellulose filters by the method of Southern (45). The probes used for Southern blots were either the plasmid pKHX-BH or a 2.3-kb BamHI-to-SalI fragment contained within pKHX-BH (Fig. 1). The 2.3-kb fragment was electrophoresed after separation by agarose gel electrophoresis and purified over an Elutip-d column (Schleicher & Schuell Co., Keene, N.H.) (8). Probes were labeled with [32P]dCTP and [32P]dGTP (New England Nuclear Corp., Boston, Mass.) by nick translation (27).

**Generation of deletion mutants by cotransfection.** For the construction of deletion mutants, ICP27-transformed cells were cotransfected with infectious KOS DNA and linearized p123ABS by the transfection technique of DeLuca and Schaffer (10). KOS DNA (1 μg) was coprecipitated with 1 μg of linearized p123ABS DNA in the presence of various amounts of salmon testis DNA in four independent cotransfections. Progeny virus from one of the cotransfections was plated on 3-3 cells, plaques were picked, and plaque isolates were screened for their ability to produce cytopathic effects in 3-3 cells but not in Vero cells. Isolates with this phenotype were plaque purified three times, amplified, and characterized as described below.

**DNA isolation.** Virion-associated, infected-cell, and transformed-cell DNAs were isolated as described by DeLuca et al. (8, 9).

**Analysis of infected-cell polypeptides.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of radioactively labeled infected-cell lysates was performed by the method of Laemmli (24) as modified by Manservigi et al. (28). In this study, cells were infected at a multiplicity of infection of 20 PFU per cell and incubated with the quantities of [35S]methionine (New England Nuclear) and for the times indicated.

**Viral DNA phenotypes.** Viral DNA phenotypes of mutants were determined in Vero cells infected with 20 PFU of each virus per cell. Infected cells were incubated at 37°C and labeled from 5 to 18 h with 10 μCi of [3H]thymidine per ml. DNA extracts were prepared and analyzed by CsCl density gradient centrifugation by the method of Aron et al. (1), except that 200 μg of proteinase K per ml was used instead of pronase.

**Nuclear runoff assays.** Nuclear runoff transcription analysis was performed as described previously (19, 47). Single-stranded M13 phase DNA (1 μg) containing HSV-1 DNA sequences complementary or anticomplementary to the indicated mRNA species (see Fig. 6) was immobilized on nitrocellulose filters. The M13 DNAs containing ICP4, 8, and 5 and gC DNA sequences were kindly provided by David Knipe (Harvard Medical School) and were described previously (16). Those containing ICP0, ICP22, and ICP27 DNA sequences were generated by W. Sacks as described previously (16). DNA probes bound to nitrocellulose filters were hybridized with [32P]labeled run-on RNA products generated from nuclei isolated from 3 x 10^6 Vero cells at 6 h postinfection.

**RESULTS**

**Generation of a cell line expressing ICP27.** Since ICP27 has been shown to be essential for virus growth (41), a cell line expressing the wild-type ICP27 gene was generated to serve as the permissive host for the derivation, propagation, and assay of ICP27 deletion mutants. For this purpose, a plasmid (pBHneo) was constructed that contained the neomycin resistance gene under control of the simian virus 40 early promoter (pSV2neo) linked to the ICP27-containing BamHI-HpaI fragment from the plasmid pKHX-BH (Fig. 1). The BamHI-HpaI fragment was selected for transformation because (i) it contains the entire ICP27 gene as well as essential 5′ regulatory sequences, and (ii) this fragment provides no sequence homology to the left of the BamHI site with plasmid p123ABS used for mutant isolation. The absence of such homology prohibits the generation of wild-type virus by recombination between the superinfecting deletion mutant and resident ICP27 sequences.

Vero cells transfected with the hybrid plasmid pBHneo were grown in the presence of G418. The resulting G418-resistant colonies were picked and amplified, and G418-resistant lines were screened for their ability to host the ICP27 mutant tsY46 at the nonpermissive temperature (39°C). Of 40 G418-resistant cell lines isolated, 7 (~6%) were able to support the replication of tsY46 at 39°C. One cell line, 3-3, was selected as the permissive host line because it demonstrated more efficient complementation of tsY46, resulting in visibly larger plaques than other cell lines at 39°C. 3-3 cells were analyzed by Southern blot analysis and shown...
FIG. 2. Southern blot analysis of ICP27-specific sequences in DNA of the G418-resistant cell line 3-3. DNA (10 μg) from Vero and 3-3 cells was digested with BamHI and Sacl and electrophoretically separated in a 0.7% agarose gel. The gel was blotted and probed with the 3P-labeled 2.3-kb BamHI-Sacl fragment contained within pKHX-BH (Fig. 1). The standards representing 3, 10, and 30 copies of the 2.3-kb ICP27-containing fragment per 3 × 109 base pairs are 60, 270, and 600 pg, respectively, of pKHX-BH (6.1 kb) cut with BamHI and Sacl.

To contain approximately one copy of the ICP27 gene per haploid genome equivalent (Fig. 2).

To assess the ability of 3-3 cells to provide biologically active ICP27 in trans, we infected these cells with tsY46 at the permissive (34°C) and nonpermissive (39°C) temperatures. Vero cells and the KOS wild-type virus were included as controls. In Vero cells at 34°C, tsY46 grew nearly as efficiently as did the wild-type virus (Table 1). At 39°C, however, growth of tsY46 in Vero cells was greatly impaired relative to KOS. In 3-3 cells at 34°C, both viruses grew equally well, and at 39°C the yield of tsY46 was only slightly lower than that of KOS, indicating that 3-3 cells expressed biologically active ICP27.

**Isolation of ICP27 deletion mutants.** Having demonstrated the suitability of 3-3 cells to serve as permissive host cells, we next attempted to generate ICP27 deletion mutants by using these cells. The plasmid p123ABS (Fig. 1) containing an engineered 1.2-kb deletion in the ICP27 gene was linearized with EcoRI and coexpressed with infectious KOS DNA. Monolayers of 3-3 cells were cotransfected with the mixture, and samples of viral DNA from transfected cultures were analyzed by Southern blot hybridization to ensure that the fragment containing the deleted form of the ICP27 gene was represented among progeny genomes. Both the wild-type and deleted forms of the ICP27-containing fragment were present in progeny DNA of all transfections (data not shown). Progeny virus from one transfected culture was plated on permissive 3-3 cells, plaques were picked, and plaque isolates were amplified and tested for their ability to produce cytopathic effects in 3-3 and Vero cells. Of 10 plaque isolates, 2 (3d1/2.2 and 5d1/2.2) produced cytopathic effects in 3-3 cells but not in Vero cells.

**Southern blot analysis of the DNA of the two mutants (Fig. 3) demonstrated that both contained the engineered 1.2-kb deletion present in p123ABS.** By contrast, a plaque isolate that grew on both 3-3 and Vero cells (i.e., an isolate that was phenotypically wild type) contained the wild-type NruI fragment. Both mutants were plaque purified three times in 3-3 cells, and a concentrated stock of each was prepared in 3-3 cells. Stocks of approximately 109 PFU/ml were prepared; as anticipated, no recombinants were detected when these stocks were assayed on Vero cells.

**Phenotypic analysis of ICP27 deletion mutants.** (i) **Growth properties.** The yields of the mutants when grown in Vero cells and assayed in either Vero or 3-3 cells were negligible (Table 2); the low titers of mutant virus detected when assays were conducted in 3-3 cells likely represent unadsorbed inoculum. By contrast, the yields of the mutants grown and assayed in 3-3 cells were similar to those of KOS in these cells, and plaque sizes were only slightly similar to those of KOS in these cells, and plaque sizes were only slightly smaller, demonstrating that the ICP27-transformed cell line complemented both 3d1.12 and 5d1.12 efficiently. Again, wild-type recombinants were not observed when ICP27 deletion mutants were grown in 3-3 cells and assayed on Vero cells.

(ii) **Polypeptide synthesis.** The polypeptide phenotypes of 3d1.12 and 5d1.12 were compared in Vero and 3-3 cells in long-labeling experiments. Included in these tests were tsY46 (a temperature-sensitive mutant in ICP27; 42), d21 (a deletion mutant in ICP8 that is DNA negative in Vero cells; 35), and KOS.

Under permissive conditions all mutants except d21 ex-
TABLE 2. Growth and plating efficiency of 3d11.2, 5d11.2, and KOS in Vero and 3-3 cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cells used for assay</th>
<th>Yield (PFU/ml) when grown in:</th>
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<tr>
<td></td>
<td></td>
<td>Vero cells</td>
</tr>
<tr>
<td>KOS</td>
<td>Vero</td>
<td>1.8 x 10^8</td>
</tr>
<tr>
<td></td>
<td>3-3</td>
<td>9.0 x 10^7</td>
</tr>
<tr>
<td>3d11.2</td>
<td>Vero</td>
<td>&lt;10^7</td>
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<tr>
<td></td>
<td>3-3</td>
<td>1 x 10^7</td>
</tr>
<tr>
<td>5d11.2</td>
<td>Vero</td>
<td>&lt;10^5</td>
</tr>
<tr>
<td></td>
<td>3-3</td>
<td>1.6 x 10^3</td>
</tr>
</tbody>
</table>

* Monolayers of Vero or 3-3 cells were infected at a multiplicity of 2.5 PFU/cell and incubated at 37°C for 18 h. Infected cultures were frozen, thawed, sonicated, and clarified by low-speed centrifugation. Supernatant fluids were then assayed in monolayers of Vero and 3-3 cells.

** When 3d11.2 and 5d11.2 were grown in 3-3 cells and assayed in Vero cells, monolayers exhibited uniform nonspecific cytopathic effects at low dilutions. This was likely due to cell killing at high multiplicity of infection.

hibited profiles similar to that of KOS (Fig. 4A). The d21 profile in permissive U-47 cells was essentially wild type, except that the level of wild-type-size ICP8 (provided by U-47 cells) was decreased, and a truncated form of ICP8 that migrated faster than pgB was synthesized by the mutant (34). The wild-type-like profiles of 3d11.2 and 5d11.2 in 3-3 cells reflect the efficiency with which these mutants are complemented in these cells.

Under nonpermissive conditions (Fig. 4B) relative to KOS, the polypeptide phenotypes of 3d11.2 and 5d11.2 were characterized by (i) the absence of a band corresponding to ICP27, (ii) higher-than-wild-type levels of two early proteins, ICP6 and ICP8; (iii) underrepresentation of pgB, gB, and the late (γ1) polypeptides ICP5 and ICP25; and (iv) the absence of the late (γ2) polypeptides ICP19 and ICP20. The immediate-early protein ICP4 was not overproduced in this study as it had been in studies of ICP27 ts mutants at the nonpermissive temperature (41).

**FIG. 4.** Polypeptide profiles of ICP27 deletion mutants grown under permissive (A) and nonpermissive (B) conditions. The KOS wild-type virus and d21, a mutant with a deletion in ICP8 rendering the virus DNA negative, were included as controls. Cells were infected with 20 PFU of the indicated virus per cell, labeled with 20 μCi of [35S]methionine per 35-mm plate from 5 to 18 h postinfection, and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The positions of immediate-early (ICP27), early (ICP19 and ICP20), and late (γ1, ICP5 and ICP25; γ2, ICP19 and ICP20) polypeptides are indicated, as are the positions of pgB and gB. Cell types and infecting viruses are shown at the top, and incubation temperatures are shown at the bottom.
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The polypeptide profiles of 3d1.2, 5dl1.2, and tsY46 under nonpermissive conditions exhibited qualitative similarities in the synthesis of early and late proteins (Fig. 4B). Quantitatively, however, tsY46-infected cells exhibited greatly reduced levels of the early proteins ICP6 and ICP8 relative to those in 3dl1.2 and 5dl1.2, whereas the three mutants induced equal but reduced amounts of the γ1 protein ICP5 relative to that in wild-type virus. The differences in the levels of synthesis of early proteins are not likely a consequence of differences in incubation temperature (37°C for deletion mutants and 39°C for tsY46), as demonstrated by the similarities in the intensities of all other viral and cellular bands.

Aside from the synthesis of a truncated form of ICP8 in Vero cells infected with the DNA-negative mutant d21, the major differences in the polypeptide profiles of 5dl1.2-, 3dl1.2-, and d21-infected cells were decreased levels of ICP6 and ICP8 in d21-infected cells. The three mutants in ICP27 were less efficient in shutting off host cell protein synthesis than wild-type virus, whereas d21 was somewhat more efficient.

From these studies it can be concluded that the 1.2-kb deletion in 3dl1.2 and 5dl1.2 results simultaneously in their inability (i) to induce the synthesis of detectable ICP27, (ii) to down-regulate early gene expression (i.e., ICP6 and ICP8), and (iii) to induce wild-type levels of late viral proteins of the γ1 and γ2 classes as well as factors involved in shut-off of host cell polypeptide synthesis. The absence of detectable ICP27 was not accompanied by overproduction of ICP4 in these experiments.

(iii) Kinetics of polypeptide synthesis. To examine the kinetics of polypeptide synthesis in nonpermissive cells, 5dl1.2- and KOS-infected Vero cells were pulse-labeled for 30 min at selected times postinfection with [35S]methionine in the presence and absence of the DNA synthesis inhibitor phosphonoacetic acid (PAA).

In the absence of PAA (Fig. 5A), the rates of synthesis of early proteins ICP6, 8, and 36 increased as a function of time through 8 h postinfection in both 5dl1.2- and KOS-infected cells. At 12 h postinfection, rates of early protein synthesis remained high in 5dl1.2-infected cells but dropped significantly in KOS-infected cells. Thus, in 5dl1.2-infected cells, early protein synthesis was not down-regulated.

With regard to late protein synthesis, the rates—and consequently the amounts—of γ1 proteins (ICP5, 25, and 44) synthesized in 5dl1.2-infected cells were markedly lower than those seen in KOS-infected cells for all labeling periods. Notably, the γ2 proteins (ICP1/2, 15, 19, 20, and 48) were not

FIG. 5. Polypeptide profiles of pulse-labeled Vero cells infected with KOS or 5dl1.2 in the presence (B) and absence (A) of the DNA synthesis inhibitor PAA (400 μg/ml). Cells were infected with 5dl1.2 or KOS at a multiplicity of 20 PFU/cell at 37°C, labeled with 100 μCi of [35S]methionine per 35-mm plate for 30 min at 2, 5, 8, and 12 h postinfection, and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (The 12-h KOS profile is indistinguishable from the 8-h KOS profile in other gels.)
detectable in 5d11.2-infected cells, whereas their levels increased with time in KOS-infected cells. (Note that the band in 5d11.2 lanes in the position of the ICP1/2 proteins in KOS lanes is a cellular protein. This is better seen in higher percentage gels.)

The glycoprotein gB and its precursor pgB have been variously classified as early (β), delayed-early, or late (γ1) proteins. Of particular interest was the observation that the rate of synthesis of pgB in cells infected with 5d11.2 and with KOS increased equally from 2 to 12 h postinfection, whereas this was not the case for the mature form of the glycoprotein (gB), which increased with time in KOS-infected cells but not in 5d11.2-infected cells. This observation strongly suggests that the activity of ICP27 is required—directly or indirectly—for either the expression of factors involved in the processing of pgB to gB or in the stabilization of gB. The failure to detect larger quantities of pgB and gB in mutant-infected cells in long-term labeling experiments (Fig. 4B) may reflect the instability of these proteins over the 4- to 18-h labeling period in cells infected with the mutants but not in cells infected with KOS. The observation that the rate of pgB synthesis was approximately equal in 5d11.2- and KOS-infected cells in pulse-labeling experiments, whereas the rates of synthesis of γ1 proteins (ICP5, 25, and 44) were markedly reduced in mutant-infected cells, distinguishes pgB from the γ1 proteins mechanistically based on the requirement of the latter proteins for ICP27.

As in long-term labeling experiments, pulse-labeling experiments with 5d11.2-infected cells revealed (i) no detectable ICP27 peptide, (ii) failure to down-regulate early genes (ICP6 and ICP8) expression, and (iii) decreased synthesis of late proteins and factors involved in shutoff of host protein synthesis.

In the presence of PAA (Fig. 5B), the patterns of viral protein synthesis in 5d11.2- and KOS-infected Vero cells were nearly identical, suggesting that the primary effect(s) of the absence of ICP27 may occur at the level of viral DNA synthesis. (The 12-h KOS profile was indistinguishable from the 8-h KOS profile in other gels.) This observation is consistent with a major block in late (γ1 and γ2) but not in early (β) protein synthesis. Arguing against this possibility, however, is the fact that substantial quantities of viral DNA (50 to 100%) were induced by the ts mutants at 39°C (41). The block in viral DNA synthesis due to the presence of early proteins (e.g., ICP6 and ICP8) in 5d11.2- and 5d11.2-infected cells (Fig. 4B and 5A) suggested that the mutants should induce the synthesis of viral DNA. We therefore assessed the levels of viral DNA present in 5d11.2- and KOS-infected Vero cells labeled from 5 to 18 h postinfection. Quantification of the CsCl gradient profiles demonstrated that the quantity of viral DNA detected in 5d11.2-infected cells was only 18% of that detected in wild-type virus-infected cells (data not shown). This finding demonstrates a requirement for ICP27 in achieving wild-type levels of viral DNA—but not an absolute requirement for ICP27 in viral DNA synthesis. Interestingly, 5d11.2 was also somewhat less efficient than KOS in turning off cellular DNA synthesis, a finding consistent with the inefficiency of the mutants to shut off cellular protein synthesis (Fig. 4B and 5A).

(v) Transcription of viral genes: nuclear runoff assays. The effect of ICP27 on the transcription of the HSV genome during productive infection has not been addressed in previous studies. Because previous studies of ts mutants in ICP27 suggested that temperature-sensitive forms of ICP27 polypeptide exhibit differing abilities to regulate viral gene expression (41), the ts mutants were not suitable tools with which to address this question. The availability of null mutants in this gene provides a more suitable tool.

In these studies, transcription of selected viral genes representing immediate-early (ICP0, 4, 22, and 27), early (ICP8), and late (γ1, ICP5; γ2, gC) genes was compared in nuclei of 5d11.2- and KOS-infected cells at 5 h postinfection in the presence and absence of PAA (Fig. 6). In the absence of PAA, major differences were observed in the rates of transcription of individual genes as well as in the extent of transcription from the antisense strand in 5d11.2-infected nuclei relative to KOS-infected nuclei (Fig. 6). Thus, significant reductions in the levels of transcription of ICP0 and ICP4 but not ICP22 were seen in mutant-infected nuclei. A minor reduction was observed in the rates of transcription of ICP0 in cells infected with 5d11.2, whereas significant reductions in ICP5 (γ1) and gC (γ2) transcription were observed in 5d11.2-infected cells. That the pattern of transcription observed in 5d11.2-infected cells is characteristic of transcription seen in cells infected with wild-type virus in the presence of inhibitors of viral DNA synthesis is demonstrated by the similarities between the patterns shown in Fig. 6. The slightly increased rates of transcription of all test genes in the case of 5d11.2 in the absence of PAA is likely due to the fact that some viral DNA synthesis occurs in these cells (i.e., 18% of wild-type DNA synthesis).

The results of nuclear runoff assays correspond with the results of assays of viral polypeptide synthesis in mutant-infected cells—especially with regard to the reduction in later protein synthesis. Since alterations in transcription likely precede viral DNA synthesis, the reduced rates of transcription characteristic of 5d11.2-infected cells are likely responsible for the reduced level of viral DNA in mutant-infected cells. Therefore ICP27—although not absolutely essential for viral DNA replication—is required to achieve wild-type levels of viral DNA synthesis.
DISCUSSION

ICP27 is essential for virus replication. The existence of four temperature-sensitive mutants of HSV-1 KOS able to replicate at 34°C but not at 39°C and whose ts mutations map to ICP27 coding sequences prompted us to conclude that ICP27 is essential for virus replication (41). Citing the replication competence of an HSV-1 17 mutant, whose mutation lies outside ICP27 but which expresses reduced yet detectable levels of ICP27, Maclean and Brown concluded that ICP27 is not essential for virus growth (25). This conclusion was based on studies of a virus whose genotype was not well defined, and no effort was made to determine the actual level of ICP27 required for virus replication. Hence, rather than demonstrating that ICP27 is not essential for virus replication, the study of Maclean and Brown suggests that low levels of ICP27 are adequate for virus growth.

In the present study, deletion of the 1.2-kb BamHI-Sall fragment (map coordinates 0.745 through 0.753) from the viral genome yielded mutants that synthesized no detectable ICP27 and were unable to replicate in Vero cells but were able to replicate efficiently in ICP27-expressing 3-3 cells. These observations and the absence of evidence indicating that other viral genes overlap ICP27 coding sequences (D. McGruer, personal communication) support the concept that ICP27 is an essential replicative protein as previously reported (41).

Functions of ICP27 in transient expression assays. Data from two types of studies suggest a role for ICP27 in the regulation of HSV gene expression: (i) transient expression assays and (ii) phenotypic studies of ICP27 ts mutants. Before attempting to compare and interpret the results of studies with ICP27 deletion mutants, a brief summary of data from transient expression assays is warranted since these assays indicate what effects ICP27 is capable of exerting on viral gene expression.

In general, ICP27 alone has failed to affect expression of cotransfected HSV genes (10, 13, 30), the exception being the recent demonstration by Rice and Knipe that ICP27 is able to transduce a chimeric gB promoter-chloramphenicol acetyltransferase gene (40). Given the limited number of viral promoters tested to date, however, future studies may reveal the existence of other ICP27-inducible target genes. More abundant evidence exists demonstrating that ICP27 can both enhance and repress expression of HSV-1 immediate-early, early, and late promoters driven by ICP4 and ICP0. Thus, ICP27 has been reported to down-regulate its own expression when this expression is induced by ICP4 and ICP0 (15). Similarly, repression by ICP27 of ICP4- and ICP0-induced expression has been documented by S. A. Rice, L. Su, and D. M. Knipe for the early ICP8 promoter (personal communication), and by Sekulovich, et al. for the tk promoter (43a). By contrast, ICP27 is able to further enhance ICP0- and ICP4-induced expression from the gB (40) and ICP5 (14, 43a) promoters. ICP27 had no effect on ICP0- and ICP4-induced expression from the gC promoter (44).

Two points can be made from these studies: (i) that ICP27-regulatory activities must be viewed in the context of other virus-regulatory activities (i.e., those of ICP4 and ICP0) and (ii) that ICP27 appears to possess both stimulatory and inhibitory activities for different HSV promoters. Because only one or two examples of each kinetic class of HSV promoter has been tested thus far, it is not possible to determine whether ICP27 acts differentially on the various classes of promoters or whether ICP27 actually plays a role in determining these kinetic classes in combination with other regulatory proteins and specific cis-acting promoter elements. In sum, the evidence to date from transient assays indicates that ICP27 acts to down-regulate expression of selected early genes and further up-regulate late (g) genes whose expression is induced by ICP0 and ICP4.

ICP27 null phenotype. The absence of ICP27 in deletion mutant-infected cells produced multiple effects including failure to down-regulate early gene expression, decreased expression of late genes of the g) class, and failure to express late genes of the g) class. Perhaps the most interesting phenotypic alteration was the dramatic reduction in the amount of viral DNA detected in mutant-infected cells. Indeed all the transcriptional and translation effects on viral gene expression produced in the absence of ICP27 are characteristic of the pattern of gene expression seen in the absence of viral DNA synthesis (16, 47). The transcription of the 5dl1.2 genome in the absence of PAA was qualitatively similar to that of 5dl1.2 and KOS in the presence of PAA (Fig. 6). The general quantitative increase in the transcription of the 5dl1.2 genome in the absence of PAA relative to the PAA-inhibited samples most likely reflects low levels of DNA synthesis. The polypeptide profile of 5dl1.2-infected Vero cells is also consistent with a markedly DNA-deficient phenotype (34). This was especially evident in pulse-labeling experiments.

The absence of any evidence implicating ICP27 directly in viral DNA synthesis (49) and the evidence from transient expression assays suggesting a role for ICP27 in the regulation of viral gene expression strongly suggest that the DNA-deficient phenotype is a consequence of aberrant viral gene expression rather than a direct effect on viral DNA synthesis. How, then, can we reconcile this hypothesis with the demonstrated stimulatory and inhibitory effects of ICP27 on viral gene expression?

Although the expression of ICP8 is not dependent on the activity of ICP27, it may be that ICP27 is required to stimulate expression of one or more of the other six genes required for viral DNA synthesis (49). If this were the case, it is likely that such a failure would go undetected in polyacrylamide gels. Fortunately, the identity of the gene(s) able to be stimulated by ICP27 can likely be determined in transient expression assays. Specifically, efforts to examine the effects of ICP27 on the expression of other genes involved in viral DNA synthesis warrants further study.

ICP27 has the ability to down-regulate early gene expression in transient assays, and failure to down-regulate early gene expression is characteristic of ICP27 deletion mutant-infected cells. It is possible that overexpression of an early protein or proteins involved in viral DNA synthesis has an inhibitory effect on this process. We have shown previously that the overexpression of at least one early protein over-represented in 5dl1.2-infected cells (ICP6) can have an inhibitory effect on the growth of the wild-type virus (34). This inhibitory effect may be at the level of viral DNA synthesis.

Finally, structural proteins of the g) class were underrepresented, and proteins of the g) class were not detected, in ICP27 deletion mutant-infected cells. If ICP27 were required to stimulate g) gene expression, as suggested by the results of transient expression assays, these proteins would be underrepresented (g) or absent (g) in the absence of ICP27. Since late proteins are required to process and stabilize viral DNA once synthesized, the viral DNA made in ICP27-infected cells may not be appropriately processed or stabili-
TABLE 3. Phenotypic variation among mutants in ICP27 relative to wild-type virus

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Immediate Protein synthesis*</th>
<th>Early</th>
<th>γ1</th>
<th>γ2</th>
<th>Viral DNA synthesis (% of wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5dl.2</td>
<td>=</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>18</td>
</tr>
<tr>
<td>tsY46</td>
<td>ICP4 = or ↑</td>
<td>↓</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>tsE6*</td>
<td>ICP4 = or ↑</td>
<td>↓</td>
<td></td>
<td></td>
<td>57</td>
</tr>
<tr>
<td>tsLG4</td>
<td>ICP4 = or ↑</td>
<td>↓</td>
<td></td>
<td></td>
<td>89*</td>
</tr>
</tbody>
</table>

*Arrows signify increased (↑) or decreased (↓) expression relative to wild-type virus.
* The phenotype of tsE6 is similar to that of tsE6 (6).
* Data from R. Sandri-Goldin (unpublished results).

ized. Such improperly processed, unprotected DNA might be readily degraded.

Both of the latter two possibilities are feasible based upon evidence from transient expression assays and from phenotypic studies of ICP27 deletion mutants. Theoretically, any or all of these possibilities could produce the phenotype of ICP27 deletion mutants.

Whatever the actual mechanism(s) responsible for the ICP27 null phenotype, it is reasonable to assume that the phenotype is a consequence of the absence of the stimulatory and inhibitory effects of ICP27 on other viral genes. Whether both activities reside in the same molecule remains to be determined. What is clear from transient expression assays and from studies of mutants in ICP27 is that this protein affects multiple viral functions.

Phenotypic variation among mutants in ICP27 suggests that ICP27 is multifunctional. The results of transient expression assays suggest that ICP27 has the ability to stimulate expression from some viral promoters and repress expression from others (cooperatively with ICP0 and ICP4), implying that ICP27 is a multifunctional protein. If we assume that this is also the case in the context of the viral genome, the deletion mutant 5dl.2 reflects gene expression in the absence of ICP27 and therefore in the absence of both activities. By contrast, one might expect ts mutants in ICP27 to vary in phenotype as a consequence of the functions expressed by the various forms of the protein. Table 3 summarizes qualitatively the relative levels of expression of the various classes of HSV proteins and of viral DNA in the backgrounds of ts mutants (41; this study) and of the null mutant 5dl.2 (this study). The levels of expression of these proteins from the genomes of all the ts mutants differed significantly from the levels of expression characteristic of the null mutant, implying that the ts forms of ICP27 retain activities associated with the modulation of viral gene expression. The manner in which specific mutations affect the proposed activities of ICP27 to contribute to the observed diversity of phenotypes remains to be determined. Especially noteworthy differences include the expression of early genes and of late genes of the γ1 class in the various mutant backgrounds.

ICP27 also possesses the ability to further enhance expression of γ1 genes induced by ICP0 and ICP4 in transient assays. Although this activity appears intact in the tsE5 and tsE6 forms of ICP27, it is lacking not only in 5dl.2 (as anticipated) but also in tsY46. This activity is deficient but not absent in tsLG4. Finally, none of the mutants—deletion or ts—is able to induce the expression of late genes of the γ2 class, despite the ability of the ts mutants to induce significant levels of viral DNA. Notably the observation that late protein synthesis is impaired in ts mutant-infected cells but that DNA levels remain high argues against the hypothesis suggested above that viral DNA stability may be compromised in the absence of late, stabilizing proteins. Alternatively, only certain late proteins induced by ICP27 may be involved in stabilizing viral DNA, and the ts mutants may vary in their ability to induce these proteins.

It is notable that the ts mutants induced the synthesis of considerably more DNA than did the deletion mutant—again implying that the ts forms of ICP27 specify activities sufficient for this purpose. The major phenotypic differences observed among the ts mutants involved differences in the levels of early and late (γ1 and γ2) proteins induced (6).

Whether the up- and down-regulatory activities of ICP27 are functions specified by the molecule itself or whether these activities are specified by ICP27 in conjunction with ICP0 and/or ICP4 remains to be determined. To address this question, the cloned ICP27 genes from ts mutants and a series of nonsense mutants in the cloned ICP27 gene are currently being tested for their ability to up- and down-regulate expression from selected immediate-early, early, and late (γ1 and γ2) viral promoters.

Whatever the mechanism underlying the phenotypes of available mutants in ICP27, it is likely that this protein plays a critical role in trans in distinguishing among the various classes of HSV genes.

ACKNOWLEDGMENTS

We thank Rozanne Sandri-Goldin and David Knipe for communicating the results of unpublished studies, Neal DeLuca and Weizhong Cai for helpful comments on the manuscript, and Meg Kaveny for manuscript preparation.

This investigation was supported by Public Health Service grants CA20264 from the National Cancer Institute and AI24010 from the National Institute of Allergy and Infectious Diseases. L.M. is the recipient of a postdoctoral fellowship grant from the Leukemia Society of America.

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