Herpes Simplex Virus Alpha Protein ICP27 Possesses Separable Positive and Negative Regulatory Activities

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The HSV-1 alpha (immediate-early) protein ICP27 expressed in transfected cells can activate the expression of certain HSV-1 promoters as well as inhibit the transactivated expression of others. We constructed a set of plasmids encoding mutant ICP27 molecules truncated at their carboxyl termini and used transfection assays to determine the functional properties of the mutant proteins. A polypeptide containing the amino-terminal 263 amino acid residues of ICP27 retained partial ability to activate gene expression but was unable to inhibit transactivation. Mutant proteins possessing 406 or 504 amino acids of ICP27 were unable to activate gene expression but retained full ability to inhibit transactivation. These results define two separable regulatory activities of ICP27, one positive and one negative, which can modulate gene expression in transfected cells. Immunoblot and immunofluorescence experiments were used to study the immunological reactivities and intracellular localizations of the mutant proteins. All proteins possessing the amino-terminal 263 amino acids of ICP27 reacted with an ICP27-specific monoclonal antibody and were localized to the cell nucleus. The mutant proteins, however, exhibited a number of phenotypes with regard to intranuclear localization. A mutant possessing 504 residues of ICP27 was similar to the wild-type protein in apparently localizing to all regions of the nucleus. A mutant containing 406 residues of ICP27, on the other hand, was mostly excluded from the nucleolar regions, while a 263-residue mutant was localized predominantly in the nucleoli. Thus, some aspect of ICP27 structure or function can dramatically affect its intranuclear distribution.

The expression of viral genes is tightly controlled during lytic infection of cells by herpes simplex virus type 1 (HSV-1) (2, 15, 19; for a review, see reference 35). The 70 to 80 viral genes have been classified in part according to the kinetics of their expression. The viral alpha (immediate-early) genes are expressed first, followed by the beta (early) and then the gamma (late) genes. The classification of viral genes into three groups is also based on several other criteria. Alpha genes are distinguished by the fact that they are transcribed in the absence of prior viral protein synthesis, whereas beta and gamma gene transcription requires expression of the alpha proteins. Beta and gamma genes can be distinguished from each other on the basis of the effect of viral DNA replication on their expression. Expression of beta genes is not stimulated by viral DNA replication, whereas expression of gamma genes is stimulated by viral DNA replication. Gamma genes have been subdivided on the basis of the extent of this effect; gamma-1 genes are expressed at low levels even in the absence of DNA replication, while gamma-2 genes require DNA replication for significant expression. In addition to the regulated induction of HSV-1 genes during infection, there also exist poorly understood negative regulatory mechanisms which act to turn down alpha and beta gene expression when beta and gamma genes, respectively, are activated.

Each HSV-1 gene possesses its own promoter and is transcribed by the host cell RNA polymerase II (4). The viral promoters contain recognizable TATA elements and, at least in the case of alpha and beta promoters, contain upstream sequence elements which are binding sites for cellular transcription factors (17, 18). Thus, at the level of transcription, HSV-1 must depend to a large extent on the machinery of the host cell for the expression of its genes. However, in the

absence of functional alpha polypeptides, the ordered program of HSV-1 gene expression does not occur (16). The alpha proteins presumably interact with the cellular machinery to either activate or inhibit the expression of the viral genes. Knowledge of how these viral regulatory proteins function is not only relevant to the biology of HSV-1 but may also provide some insight into how cellular gene expression can be modulated during such processes as signal transduction or development.

One alpha protein involved in viral gene regulation is ICP (infected-cell polypeptide) 27. ICP27 is a 63-kilodalton nuclear phosphoprotein expressed immediately after infection (1, 15, 20, 27, 30, 31). Evidence that ICP27 is involved in gene regulation initially came from the phenotypic analysis of a set of HSV-1 mutants with temperature-sensitive (*ts*) mutations in the ICP27 gene (36). At the nonpermissive temperature, these mutants are unable to activate a subset of beta and gamma genes and show enhanced expression of most alpha polypeptides. The *ts* mutations in the ICP27 gene have a differential effect on expression of the later (beta and gamma) genes; one gene which is expressed quite poorly by the mutants is the glycoprotein B (gB) gene (34).

Additional evidence that ICP27 regulates gene expression comes from transient expression assays. These studies have shown that ICP27 expressed in transfected cells can alter the expression of many cotransfected HSV-1 target genes. Several studies have shown that ICP27 can induce expression of chimeric target genes driven by specific beta or gamma promoters (8, 34, 37, 39a). For some of these promoters, the effect requires the expression of the HSV-1 alpha proteins ICP4, ICP0, or both. However, expression of ICP27 alone is able to stimulate expression of the gB promoter in transfected Vero cells, indicating that at least part of the positive regulatory effect of ICP27 is independent of other viral proteins. ICP27 is also able to inhibit gene expression from

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another set of chimeric genes driven by beta or gamma promoters (37, 39a). The negative regulatory effect of ICP27 is complex in that ICP27 has little or no effect on the basal promoter activity of these genes but rather acts to counteract the stimulatory effect of the HSV-1 transactivating proteins ICP4 and ICP0.

In this study, we have constructed a set of mutant ICP27 genes that encode ICP27 molecules truncated at their carboxyl termini. Transient expression assays were used to determine the functional properties of these mutant polypeptides. Our results indicate that ICP27 is a complex protein possessing at least two separable activities having quite distinct effects on gene expression.

MATERIALS AND METHODS

Plasmids. The plasmids pBH27 (encoding ICP27 [34]), pSHZ (encoding ICP0 [28]), and pSG1 (encoding ICP4 and ICP0 [10]) have been previously described. The plasmids pK1-2, which encodes ICP4 (7), and pIE3-CAT (6) were gifts from Neal DeLuca. The plasmid pgB-CAT(−175) contains the HSV-1 gB promoter (nucleotides −175 to +41 relative to the transcriptional start site) fused to the chloramphenicol acetyl transferase (CAT) gene and was previously called pgB-CAT-5′del(−175) (34). The plasmid p8-CAT contains the HSV-1 ICP8 promoter (nucleotides −344 to +91) linked to the CAT gene (39a). The plasmid pgC-CAT was derived in the following manner. A 310-base-pair AvaII restriction fragment containing nucleotides −248 to +62 relative to the transcriptional start site of the gC gene (14) was treated with the Klenow fragment of Escherichia coli DNA polymerase to fill in the 3′-recessed ends (23), and HindIII linkers (New England BioLabs, Beverly, Mass.) were ligated on. After digestion with HindIII, the DNA was inserted into the HindIII site of pSVOd(ori⁻)-CAT (3). A transformant carrying the gC promoter inserted correctly relative to the CAT gene was isolated.

The mutated derivatives of pBH27 (Fig. 1) were constructed in the following ways. The plasmid pBH-504R was derived from the plasmid pPs27pd1, which contains a 6.1-kb PstI fragment containing the ICP27 gene derived from the HSV-1 strain KOS1.1 cloned into pUC19. pPs27pd1 additionally contains a deletion in the pUC19 polylinker region which deletes restriction sites between the PstI and EcoRI sites. pPs27pd1, which contains an SspI site in the ICP27-coding region as well as one in the vector sequences, was linearized by partial digestion with SspI. A 14-nucleotide XbaI linker containing stop codons in all three reading frames, CTAGTCTAGACTAG (New England BioLabs), was ligated on. The DNA was restricted with XbaI, religated, and used to transform E. coli. A transformant, pPs-504R, containing the linker in the ICP27-coding region was isolated. The plasmid pBH-504R was derived by subcloning the 2.4-kb BamHI-HpaI insert containing the mutated ICP27 gene into the BamHI and HincII sites of pUC19. The plasmid pBH-406R was obtained by first cloning the XbaI termination linker into the StuI site of pPs27pd1 to create the plasmid pPs-406R. The 2.4-kb BamHI-HpaI fragment was subcloned into the BamHI and HincII sites of pUC19 to make pBH-406R. Construction of the plasmid pBH-263R (formerly called pBHX1), which contains the XbaI termination linker at the SalI site of the ICP27 gene, was described previously (39a). The plasmid pBH-59R was constructed by restricting pBH-263R with RsrII, which cuts twice within the ICP27-coding sequences. The 3′-recessed ends were repaired with the Klenow fragment, and an NheI linker con-
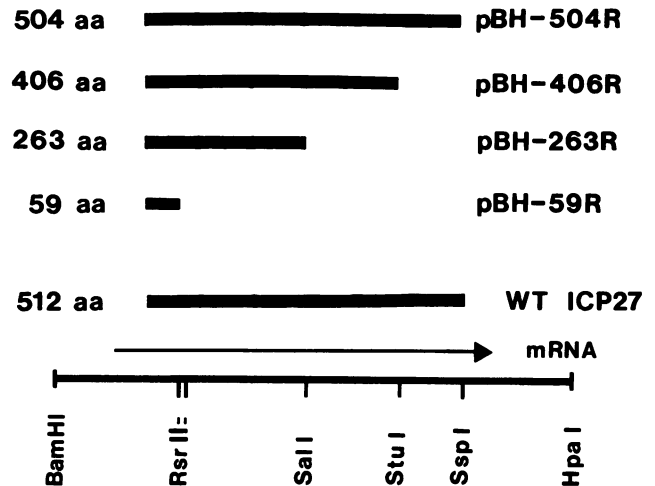

FIG. 1. Schematic representation of wild-type and mutant ICP27 polypeptides. ■■, Polypeptides encoded by the wild-type and mutant plasmids. To the left are shown the numbers of ICP27 amino acid residues (aa) identical to ICP27 in each protein. The 59R polypeptide contains one additional carboxyl-terminal amino acid, while the 504R mutant polypeptide contains four. The restriction sites used to construct the various mutant plasmids are indicated at the bottom of the figure, as is the location of the sequences encoding the ICP27 mRNA.

taining stop codons in all three reading frames, CTAGCTAGCTAG (New England BioLabs), was ligated on. After digestion with NheI, religation, and transformation into E. coli, an isolate was obtained, pBH-59R, which contained the NheI linker inserted at the site of the small RsrII restriction fragment deletion.

The mutant ICP27 plasmids were named on the basis of the number of amino acid residues of wild-type ICP27 which are predicted to be encoded by the mutant plasmid. The insertion of the stop codon linkers into the ICP27-coding region in some cases would be expected to add additional amino acid residues derived from the linker DNA onto the carboxyl terminus of the truncated protein. These can be predicted from the sequence of the linkers and the known sequence of the ICP27 gene (25). In the cases of pBH-263R and pBH-406R, which encoded the 263R and 406R proteins, the linker DNA addition restored one wild-type codon followed by a stop codon so that no new additional amino acids were added. In the cases of pBH-59R and pBH-504R, the linker addition was predicted to create one and four novel carboxyl-terminal codons, respectively. Thus, pBH-59R was predicted to encode a 60-amino-acid polypeptide (59R) with a carboxyl-terminal serine residue, and pBH-504R was predicted to encode a 508-amino-acid polypeptide (504R) with carboxyl-terminal serine-serine-leucine-aspartate residues.

Transfections and CAT assays. Vero cells were transfected by using the calcium phosphate precipitation procedure as previously described (12, 34). For immunoblot analysis, CAT assays, and complementation experiments, transfections were done with 25-cm² culture flasks. Carrier pUC19 DNA was always added to bring the total amount of DNA to 16 or 18 μg. For immunofluorescence studies, transfections were done with cells on 12-mm circular cover slips; 0.5 to 1.5 μg of pBH27 or its mutated derivatives and enough pUC19 DNA to bring the total amount of DNA to 6 μg were added. Cells transfected for CAT assays were harvested 40 to 48

h later. CAT assays were performed and quantitated as previously described (34).

Immunoblot and immunofluorescence assays. For immunoblot analysis, transfected cells were harvested after 2 days. Equal fractions of the cell lysates were separated by sodium dodecyl sulfate-gel electrophoresis (21) and electrophoretically transferred to a nitrocellulose filters at 30 V and 0.2 A for 12 to 16 h in a Transblot cell (Bio-Rad Laboratories, Richmond, Calif.). The filter was pretreated for 30 min at room temperature in TBST (10 mM Tris hydrochloride, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 1% bovine serum albumin and then treated with a 1:200 dilution of ICP27-specific monoclonal antibody H1113 (1) in TBST for 30 min. The filter was then washed three times for 5 min in TBST and treated with a 1:6,000 dilution of anti-mouse immunoglobulin G alkaline-phosphatase conjugate (Promega Biotec, Madison, Wis.) in TBST for 30 min and then washed again as before. Alkaline phosphatase activity was detected by a color reaction upon incubating the filter in 100 mM Tris hydrochloride, pH 9.5–100 mM sodium chloride–5 mM magnesium chloride–300 µg Nitro Blue Tetrazolium per ml–150 µg of 5-bromo-4-chloro-3-indolyl phosphate per ml.

For immunofluorescence, cells were processed 1 day after transfection. The cells were fixed and permeabilized as described previously (32). The cells were incubated with a 1:200 dilution of H1113 and then with a 1:100 dilution of rhodamine-conjugated goat anti-mouse immunoglobulin antibody (Cooper Biomedical, Inc., West Chester, Pa.).

Complementation assay. Vero cells were transfected at 37°C with 8 µg of wild-type or mutant ICP27 plasmid. At 28 h posttransfection, the cells were infected with 2 PFU of the HSV-1 ICP27 mutant d27-1 (S. Rice and D. Knipe, unpublished results) per cell. This virus does not grow in Vero cells because of a 1.6-kilobase BamHI-StuI deletion which deletes the ICP27 gene promoter and 80% of the coding region. The infections were incubated at 34°C for 40 h and then harvested. Virus titers were determined by plaque assay on V27 cells, a derivative of Vero cells which contains an integrated copy of ICP27 and complements d27-1 for growth (S. Rice and D. Knipe, unpublished results).

RESULTS

Expression and nuclear localization of mutant ICP27 polypeptides. We and others have previously shown that ICP27 expressed in transfected Vero cells can activate the expression of certain HSV-1 promoter-regulatory regions as well as repress the transactivated expression of a number of others (8, 34, 37, 39a). These two apparently distinct effects of ICP27 could in theory be due to one regulatory activity of the polypeptide. For example, the position in a promoter of a DNA sequence element responsive to a single ICP27 activity might determine whether the activity had a positive or negative effect on gene expression. Alternatively, the ICP27 polypeptide might possess two activities, one which activates expression of particular genes and one which represses expression of others. In this case, it should be possible to genetically separate the two activities of ICP27. To test this, we constructed a set of plasmids that encode mutant ICP27 polypeptides truncated at their carboxyl termini (Fig. 1). This was accomplished by inserting an oligonucleotide linker containing nonsense codons in all three reading frames into various sites of pBH27, a plasmid which encodes the 512-amino-acid-residue ICP27. In the case of the mutant pBH-59R, a small deletion of 35 base pairs was also made. The end result was a set of mutant plasmids very similar in size
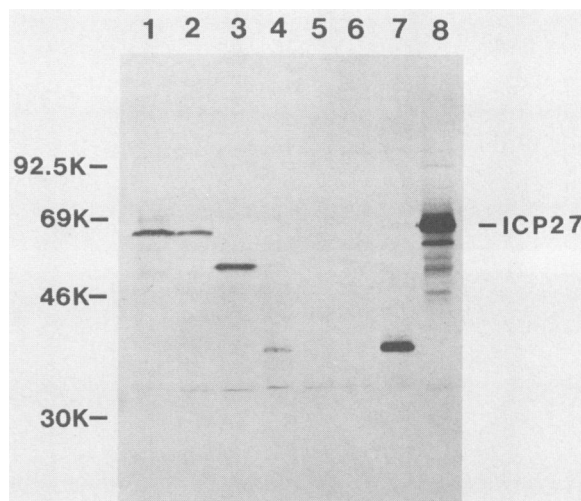

FIG. 2. Immunoblot analysis of mutant ICP27 polypeptides. ICP27-related proteins expressed in transfected (lanes 1 through 6) or infected (lane 7 and 8) Vero cells were detected by immunoblotting with the H1113 antibody. The plasmids transfected were pBH27 (lane 1), pBH-504R (lane 2), pBH-406R (lane 3), pBH-263R (lane 4), pBH-59R (lane 5), and pUC19 (lane 6). The viral strains used for infection were n263R (lane 7) and wild-type HSV-1 strain KOS1.1 (lane 8). The position of the wild-type ICP27 is indicated on the right. The molecular sizes of the ^{14}C-labeled standards are indicated on the left in kilodaltons (K).

and structure but predicted to encode truncated polypeptides containing 504, 406, 263, or 59 amino-terminal amino acids derived from ICP27. The 59R protein was predicted to contain one additional carboxyl-terminal amino acid derived from the linker sequence, while the 504R protein was predicted to contain four additional amino acids.

To test whether the mutant genes expressed proteins of the predicted size, wild-type pBH27 or the mutant plasmids were transfected into Vero cells. Cell extracts prepared 2 days after transfection were subjected to sodium dodecyl sulfate-gel electrophoresis, and the separated proteins were transferred to a nitrocellulose filter. The filter was probed with H1113, a monoclonal antibody directed against HSV-1 ICP27 (1) (Fig. 2). Both the wild-type pBH27 (Fig. 2, lane 1) and the mutant pBH-504 (lane 2) plasmids encoded a protein which reacted with the antibody and comigrated with the 63-kilodalton (kDa) ICP27 protein made in HSV-1-infected cells (lane 8). The 504R polypeptide would be expected to comigrate with the wild-type ICP27 because it is only four amino acids shorter. The mutant pBH-406R (lane 3) expressed an ICP27-related protein with an apparent molecular mass of 53 kDa, in rough agreement with the predicted size of 45 kDa. The mutant pBH-263R (lane 4) expressed a protein of approximately 38 kDa, again in rough agreement with the predicted size of 29 kDa. Although the 38-kDa band was weak, the signal appeared to be real because a protein of the same mobility was expressed by an HSV-1 recombinant virus, n263R, in which the wild-type ICP27 gene has been replaced by the 263R mutant allele (lane 7; S. Rice and D. Knipe, unpublished results). No proteins that reacted with the antibody were detected in cells transfected with pBH-59R (lane 5) or carrier pUC19 DNA only (lane 6).

An immunofluorescence assay with the H1113 monoclonal antibody was used to examine the cellular localization of ICP27 and its truncated derivatives in transfected Vero cells. A low percentage (roughly 1%) of the cells transfected with
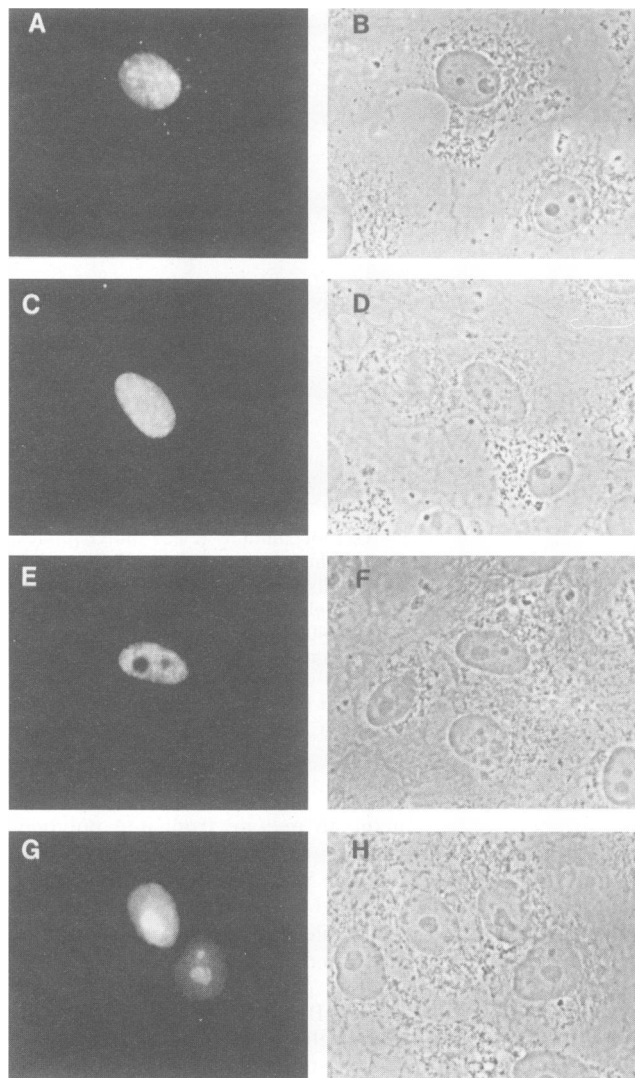
FIG. 3. Nuclear localization of wild-type and mutant ICP27 polypeptides in transfected cells. Vero cells were transfected with pBH27 (A and B), pBH-504R (C and D), pBH-406R (E and F), and pBH-263R (G and H). The cells were stained for immunofluorescence using the H1113 antibody. Panels A, C, E, and G are immunofluorescence micrographs, while panels B, D, F, and H are the corresponding phase-contrast micrographs.

FIG. 4. Ability of ICP27 mutant proteins to transactivate expression of a gB-CAT hybrid gene. Vero cells were transfected with 9 μg of pgB-CAT(−175) and the indicated amounts of plasmids encoding wild-type or mutant ICP27 molecules. The ICP27 plasmids transfected were wild-type (WT) pBH27 (O), pBH-59R (●), pBH-263R (△), pBH-406R (▲), or pBH-504R (□).

pBH27 expressed the wild-type ICP27 (Fig. 3A and B). In these cells, the protein was clearly localized to the nucleus. Cells transfected with the pBH-504R, pBH-406R, and pBH-263R plasmids also showed nuclear fluorescence (Fig. 3C, E, and G), while cells transfected with pBH-59R were negative for antibody staining (not shown). These results suggested that the ICP27 polypeptide contains a signal within its first 263 amino acids that confers nuclear localization ability. In addition, the immunofluorescence and immunoblot data together indicated that the epitope recognized by the H1113 monoclonal antibody is contained within the first 263 amino acid residues of ICP27.

An unexpected observation was that the mutant polypeptides showed quite different phenotypes with regard to intranuclear localization. In cells transfected with the wild-type plasmid, ICP27 was localized to both the nucleolar and nonnucleolar portions of the nucleus (Fig. 3A and B),
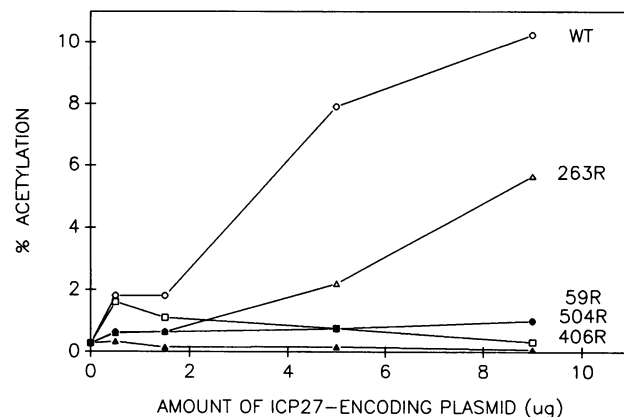
although cells could be found in which one or the other compartment showed preferential staining. Cells expressing ICP27 often showed areas of intranuclear accumulation of ICP27, but these did not correspond to the nucleoli (Fig. 3A and B). The 504R protein was very similar to wild-type ICP27 in its pattern of intranuclear localization (Fig. 3C and D). The 406R protein, however, exhibited a general inability to localize efficiently to the nucleolar compartment of the nucleus (Fig. 3E and F), although a small percentage of the positive cells (<20%) showed some nucleolar staining (not shown). Nearly all the cells expressing the 263R protein, on the other hand, showed preferential localization of the truncated ICP27 molecule to the nucleoli, with the rest of the nucleus staining more weakly (Fig. 3G and H). These results indicate that some aspect of ICP27 structure or function can dramatically influence the ability of the protein to localize within specific compartments of the cell nucleus.

Ability of the mutant polypeptides to transactivate gene expression. Wild-type ICP27 can transactivate expression of a gene containing the HSV-1 gB promoter fused to the CAT gene in transfected Vero cells (34). To determine whether any of the truncated ICP27 molecules could also transactivate gB-CAT, we transfected a constant amount of the plasmid pgB-CAT(−175) with increasing amounts of wild-type or mutant ICP27 plasmid. CAT activities were determined in extracts prepared 2 days after transfection. As expected, the wild-type ICP27 greatly stimulated expression of the gB-CAT chimeric gene (Fig. 4). Among the mutant polypeptides, only the 263R protein was able to stimulate CAT expression, although less efficiently than wild-type ICP27. This result is consistent with an earlier analysis in which the pBH-263R plasmid (previously called pBHX1) showed residual ability to stimulate gB-CAT expression (34).

ICP27 is able to work in concert with ICP4, ICP0, or both to stimulate expression from the HSV-1 alkaline exonuclease gene or ICP5 gene promoters in transfected cells (8, 37, 39a). We identified an additional HSV-1 promoter, that of the gamma-2 glycoprotein C (gC) gene, which is stimulated by ICP27 in transfected cells in the presence of either ICP4 or ICP0. Expression from the gC promoter-CAT chimeric gene was undetectable in the absence of alpha proteins (Table 1). Cotransfection of plasmids encoding either ICP27,

TABLE 1. Transactivation of a glycoprotein C-CAT chimeric
gene by HSV-1 alpha (α) proteins

Plasmid(s) transfected[a]	HSV α proteins expressed	CAT activity[b]
gC-CAT	None	0.00 ± 0.01
gC-CAT + pBH27	ICP27	0.08 ± 0.02
gC-CAT + pK1-2	ICP4	0.13 ± 0.02
gC-CAT + pSHZ	ICP0	0.22 ± 0.07
gC-CAT + pBH27 + pK1-2	ICP27 + ICP4	0.91 ± 0.12
gC-CAT + pBH27 + pSHZ	ICP27 + ICP0	3.20 ± 0.58

[a] CAT plasmid (8 μg) and alpha protein plasmids (4 μg of each) were transfected in triplicate into 25-cm² culture flasks.

[b] Values shown are the mean CAT activity (% acetylation) ± standard deviation from the mean.

ICP4, or ICP0 alone did not induce significant expression of the gC-CAT gene. However, when both the ICP27 and ICP4 plasmids were cotransfected with pgC-CAT, a synergistic induction of CAT expression was observed. A similar but more dramatic synergistic effect was observed when the ICP27 and ICP0 plasmids were cotransfected.

We determined which of the mutant ICP27 polypeptides could work in concert with ICP4 to stimulate gC-CAT expression. Vero cells were transfected with a constant amount of gC-CAT and pK1-2 (encoding ICP4) and increasing amounts of the wild-type or mutant ICP27 plasmids. As expected, ICP4 and the wild-type ICP27 efficiently induced gC-CAT expression (Fig. 5). Among the mutant polypeptides, only the 263R protein was able to consistently act with ICP4 to induce gC-CAT expression, although much less efficiently than the wild-type ICP27. Thus, the ICP27 mutant polypeptides showed the same properties with respect to transactivation of both the gB and gC promoters in that only the 263R protein retains stimulatory activity.

Ability of the mutant polypeptides to inhibit HSV-1 gene transactivation. Wild-type ICP27 can inhibit the ability of ICP4 and ICP0 to stimulate the expression of a chimeric gene in which the HSV-1 ICP8 gene promoter is linked to the CAT gene (39a). To determine which of the mutant ICP27 gene products could inhibit ICP8 promoter transactivation, we
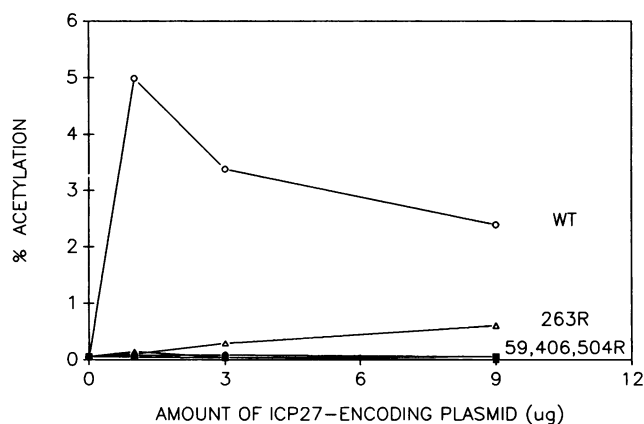

FIG. 5. Ability of ICP27 mutant proteins to transactivate expression of a gC-CAT hybrid gene in the presence of ICP4. Vero cells were transfected with 7 μg of pgC-CAT, 2 μg of pK1-2 (encoding ICP4), and the indicated amounts of plasmid encoding wild-type or mutant ICP27 molecules. The ICP27 plasmids transfected were pBH27 (○), pBH-59R (●), pBH-263R (△), pBH-406R (▲), or pBH-504R (□).

FIG. 6. Ability of ICP27 mutant proteins to inhibit the transactivated expression of an ICP8-CAT hybrid gene. Vero cells were transfected with 4 μg of p8-CAT, 4 μg of pK1-2 (encoding ICP4), and the indicated amounts of plasmid encoding wild-type or mutant ICP27 molecules. The ICP27 plasmids transfected were pBH27 (○), pBH-59R (●), pBH-263R (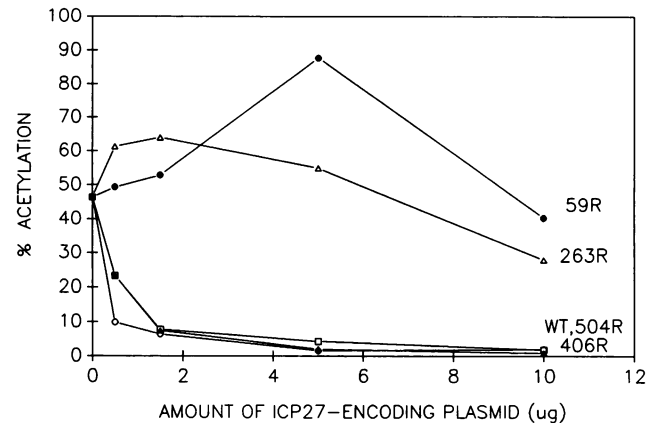△), pBH-406R (▲), or pBH-504R (□). The basal level of 8-CAT expression (no pK1-2 added) in this experiment was 2.96% acetylation.

transfected Vero cells with a constant amount of p8-CAT and pK1-2 (encoding ICP4) and increasing amounts of the wild-type or mutant ICP27 plasmids. Wild-type ICP27 and both the 406R and 504R mutant polypeptides were able to efficiently inhibit ICP4-activated expression from p8-CAT (Fig. 6). However, neither the 59R nor the 263R polypeptides were able to significantly inhibit ICP4-mediated transactivation.

These results, in combination with those of the transactivation experiments, indicated that ICP27 possesses two different activities. The 263R polypeptide retains partial ability to transactivate the gB and gC promoters but has lost the ability to inhibit ICP4-mediated transactivation of the ICP8 promoter. Conversely, the 406R and 504R polypeptides have lost the ability to transactivate gene expression but retain full ability to inhibit transactivation. Because these two activities can be distinguished genetically, we conclude that they are separable activities of the ICP27 polypeptide.

Repression of transactivated gB-CAT expression by the 406R and 504R polypeptides. The 406R and 504R polypeptides have lost the ability to transactivate gB-CAT but retain full ability to inhibit the transactivated expression of 8-CAT. This raised the question of whether these proteins could inhibit the transactivated expression of the gB-CAT chimeric gene. Consistent with previous data (34), expression of ICP4 and ICP0 transactivated gB-CAT (Fig. 7A). Furthermore, wild-type ICP27 further induced the level of expression. The 59R polypeptide had no effect on the ability of ICP4 and ICP0 to stimulate gB-CAT expression, while the 263R polypeptide had a small positive effect. This is consistent with a residual amount of transactivating ability possessed by the 263R protein. The 504R and 406R polypeptides, however, completely inhibited the ability of ICP4 and ICP0 to transactivate the gB-CAT chimeric gene. To eliminate the possibility that expression of the 406R and 504R proteins caused a relatively nonspecific inhibition of gene expression in transfected cells, we determined the effect of these proteins on the expression of another HSV-1 promoter, that of the ICP4 (or IE3) gene. This promoter, at least under some conditions, is not affected by ICP27 (39a). Cotransfection of
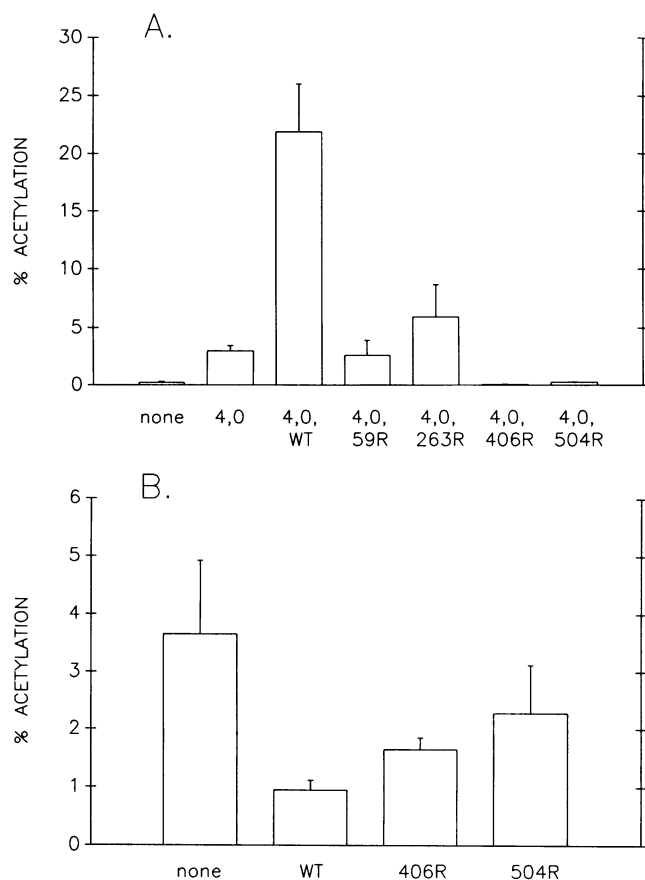
FIG. 7. Effect of ICP27 mutant proteins on the transactivated expression of a gB-CAT hybrid gene. (A) Vero cells were transfected with 8 μg of pgB-CAT(−175), 4 μg of pSG1 (encoding ICP4 and ICP0), and 4 μg of plasmid encoding wild-type or mutant ICP27 molecules. (B) Vero cells were transfected with 2 μg of pIE3-CAT and 4 μg of plasmid encoding wild-type or mutant ICP27 molecules. All transfections were done in triplicate, and the standard errors are indicated. At the bottom of each bar, the alpha proteins expressed in each situation are indicated.

TABLE 2. Ability of mutant ICP27 plasmids to complement HSV-1 ICP27 deletion mutant d27-1

Plasmid transfected[a]	Virus yield (PFU/ml)[b]	Complementation index[c]
None	2×10^2	0.4
pUC19	5×10^2	1.0
pBH27 (wt)	4×10^4	80
pBH-59R	2×10^2	0.4
pBH-263R	4×10^2	0.8
pBH-406R	8×10^2	1.4
pBH-504R	1×10^3	2.0

[a] Vero cells were transfected with the plasmids indicated. At 28 h post-transfection the cells were infected with 2 PFU of d27-1 per cell and incubated for a further 40 h at 34°C before being harvested. wt, Wild type.
[b] Determined by plaque assay on V27 cells.
[c] Expressed as virus yield relative to transfection with pUC19 DNA.

the wild-type ICP27 plasmid caused a moderate (three- to fourfold) decrease in expression of an IE3-CAT chimeric gene (6) (Fig. 7B). Cotransfection of plasmids encoding either the 406R or 504R proteins had less of an inhibitory effect, reducing IE3-CAT expression only by about twofold. Therefore, the strong negative effect of the 406R and 504R polypeptides on the transactivated expression of gB-CAT was not due to a general inhibitory effect of these proteins.

Ability of mutant plasmids to complement growth of an HSV-1 ICP27 deletion mutant. It is not clear how the two regulatory activities of ICP27 identified in transfected cells relate to functions which ICP27 performs during lytic infection. Because expression of an HSV-1 polypeptide from a transfected plasmid is able to partially complement the growth of certain HSV-1 mutants (33), including ICP27 ts mutants (36), we determined whether any of the mutant plasmids would be able to complement the growth of an HSV-1 ICP27 deletion mutant. The mutant used was d27-1, which contains a 1.6-kilobase deletion including the ICP27 gene promoter and approximately 80% of the ICP27-coding region (S. Rice and D. Knipe, unpublished results). This mutant is unable to replicate in Vero cells but grows efficiently in V27 cells, a cell line derived from Vero cells which

contains an integrated copy of the ICP27 gene and complements the growth of the mutant. Vero cells were transfected with control DNA, the wild-type ICP27 plasmid, or the mutant ICP27 plasmids. After 28 h, the cells were infected with d27-1 and allowed to undergo an infectious cycle. Virus yield was measured by plaque assay on V27 cells (Table 2). Transfection of the wild-type pBH27 plasmid increased significantly (80-fold) the yield of the mutant virus. In contrast, none of the mutant plasmids was able to significantly complement the growth of d27-1. Because the HSV-1 insert in the plasmid and the deletion in d27-1 are nonoverlapping, it is not possible to generate wild-type recombinants by homologous recombination in this experiment. Therefore, the increase in viral titer was due to complementation of ICP27 function by the transfected plasmid. The results of this experiment suggest that each mutant polypeptide is defective for a function of ICP27 required for lytic growth. However, because the 263R protein appeared to be expressed in lesser amounts in transfected cells compared with the wild-type protein (Fig. 2), we cannot exclude the possibility that this protein fails to complement d27-1 growth at least in part because of its reduced level of accumulation.

DISCUSSION

Identification of two separable ICP27 regulatory activities. Previous studies have indicated that the HSV-1 alpha protein ICP27 can either positively or negatively modulate expression of certain HSV-1 promoters in transient expression assays. In this study, similar assays were used to examine the functional properties of a set of truncated derivatives of ICP27 (summarized in Table 3). The mutant polypeptides showed three phenotypes with regard to their abilities to affect gene expression. The 504R and 406R mutants were defective for the ICP27 transactivation function but retained full ability to inhibit ICP4-mediated transactivation of the ICP8 gene promoter. The 263R polypeptide, on the other hand, retained partial transactivation activity but was defective for inhibition of ICP8 promoter transactivation. Lastly, the 59R protein was nonfunctional in both gene expression assays. These results indicate that ICP27 possesses two genetically separable activities which can modulate gene expression in transfected cells. One activity had a positive effect on gene expression, while the other inhibited gene expression. In this respect, ICP27 is similar to the adenovirus E1a proteins, which also contain two separable activities which up or down regulate gene expression in trans (26).

The conclusion that ICP27 possesses both positive and negative regulatory functions is consistent with the pheno-

TABLE 3. Summary of properties of wild-type and truncated ICP27 polypeptides in transfected Vero cells

Protein	Predicted no. of amino acid residues	Transactivation of gB-CAT and gC-CAT[a]	Inhibition of 8-CAT transactivation	Reaction to H1113 antibody	Subcellular location[b]
ICP27	512	+	+	+	N
504R	508	−	+	+	N
406R	406	−	+	+	N; nucleolar exclusion
263R	263	+/−	−	+	N; predominantly nucleolar
59R	60	−	−	−	Unknown

[a] +/−, Partial activity.
[b] N, Nuclear.

types of HSV-1 mutants containing lesions in the ICP27 gene. Viruses containing *ts* mutations in the ICP27 gene are defective at the nonpermissive temperature both for the repression of alpha gene expression and for the activation of many beta and gamma genes (34, 36). In addition, viruses containing deletions in the ICP27 locus overexpress certain viral gene products and underexpress others (24; S. Rice and D. Knipe, unpublished results). It thus seems probable that the two activities that we have identified in transfected cells correspond to distinct positive and negative functions that ICP27 performs during lytic infection.

Although our results indicate that ICP27 possesses two separable activities, we do not know whether the two activities are entirely independent. The promoter specificity of the two activities suggests that they are mediated through specific *cis*-acting DNA sequence elements in the promoters affected. The elements which mediate positive and negative regulation could be the same or different. It is of interest that the 406R and the 504R mutants can inhibit ICP4- and ICP0-mediated transactivation of the gB-CAT construct, a gene that the wild-type ICP27 transactivates. One possibility is that the gB promoter contains a single element mediating either positive or negative regulation. Alternatively, the gB promoter may contain separate positive and negative elements, with positive regulation dominating in the presence of the wild-type protein. Further understanding of the mechanisms of transactivation and transrepression by ICP27 clearly requires an identification of the *cis*-acting DNA sequences involved. It should be emphasized that although ICP27 has been reported to have some affinity for double-stranded DNA in vitro (13) it is not known whether it is associated with DNA in vivo. ICP27 could certainly affect gene expression by mechanisms other than DNA binding, for example, by altering the abundance or activity of viral or cellular regulatory factors.

It is clearly of interest to define the regions of the ICP27 polypeptide that mediate its positive and negative effects. The data regarding the transactivation function of ICP27 do not lead to a simple map for the localization of this activity. The carboxyl-terminal region of ICP27 appears to be critical for transactivation because both the 504R and 406R polypeptides are defective. Surprisingly, the 263R protein, which has a much larger carboxyl-terminal deletion, possesses partial transactivation activity. The 263R protein accumulated to lower levels than the wild-type protein, and therefore its reduced activity in the transactivation assays could be due, at least in part, to its reduced level of accumulation. In any case, it is not clear why the larger 406R and 504R polypeptides do not express any transactivating function. One possible explanation is that the carboxyl-terminal amino acids of the 504R and 406R mutants interfere with the ability of the amino-terminal portion of the protein to transactivate gene expression.

Our results regarding the localization of the negative

regulatory activity of ICP27 can be interpreted more easily. The 504R and 406R proteins retained this function, indicating that the carboxyl-terminal 106 amino acids are not required. However, truncation of the protein to 263 residues resulted in loss of activity, indicating that residues between 263 and 406 are necessary. The definition of the domains involved in the two regulatory activities of ICP27 clearly requires more extensive mutagenic analysis of the ICP27 gene.

Other herpesviruses encode proteins with recognizable amino acid homology to HSV-1 ICP27. These genes include the BMLF1 gene of Epstein-Barr virus, gene 4 of varicella-zoster virus, and the IE 52K gene of herpesvirus saimari (5, 11). The BMLF1 and IE 52K gene products can transactivate the expression of a number of target genes in transfection assays (22, 29, 40). We are unaware of any studies indicating whether these gene products can inhibit the expression of cotransfected genes. It remains to be seen whether ICP27 and the other proteins share a common mechanism for transactivation and which, if any, of the other herpesvirus proteins possess a negative regulatory activity.

Transactivation of gene expression by ICP27. We identified an additional HSV-1 promoter, that of the gamma-2 gene gC, which is transactivated in transfected Vero cells by ICP27. Similar to the HSV-1 alkaline exonuclease gene and ICP5 gene promoters (8, 37, 39a), induction of gC promoter expression by ICP27 required coexpression of either ICP4 or ICP0. Our results regarding the gC promoter differ from two previously published reports. Shapira et al. (38) failed to find an effect of ICP27 on expression of the gC promoter in transfected Vero cells, even in the presence of ICP4 and ICP0. On the other hand, Sekulovich et al. (37) reported that ICP27 can inhibit the ability of ICP4 and ICP0 to transactivate the gC promoter in transfected rabbit skin fibroblasts. These contrasting results suggest that variables such as cell type or the exact extent of the gC promoter insert may affect the response of this promoter to alpha proteins. Indeed, Everett has recently demonstrated that promoter sequence, cell type, and even transfection protocols can qualitatively alter the responses of various promoters to induction by ICP4 and ICP0 (9).

The fact that ICP27 can act by itself to stimulate gB promoter expression but in all other cases requires expression of additional alpha polypeptides raises the possibility that ICP27 might have two mechanisms for transactivating gene expression, one independent of other alpha proteins and one dependent on ICP4, ICP0, or both. The observation that the 263R protein (unlike the other mutant forms of ICP27) retains partial ability to transactivate both gB-CAT and gC-CAT suggests that there is a common mechanism by which ICP27 activates these two genes. It remains unclear why in most instances ICP27 alone is unable to induce detectable gene expression from test genes. Perhaps two or

more independent pathways of transactivation are required to initiate detectable gene expression for some promoters.

Nuclear and intranuclear localization of ICP27. The 504R, 406R, and 263R proteins all localize specifically to the cell nucleus. Thus, ICP27 may contain a signal for nuclear localization within its amino-terminal 263 amino acids. Surprisingly, the 504R, 406R, and 263R proteins showed distinct patterns of intranuclear localization. The 504R protein was similar to the wild-type protein in its apparent ability to localize to all regions of the nucleus. The 406R protein, however, showed a general inability to localize within the nucleolar regions of the nucleus. The 263R protein preferentially localized to the nucleoli. It is unclear whether there are functional consequences to these variable distributions of the mutant polypeptides in the transfected-cell nuclei. The 504R and 406R mutants are indistinguishable in their ability to affect gene expression but have different phenotypes with respect to intranuclear localization. Thus, there is no simple correlation between the ability of a mutant polypeptide to affect gene expression and its pattern of intranuclear localization. A larger collection of mutants is needed to address this question further.

The fact that the wild-type ICP27 and some of its derivatives can efficiently localize to the nucleolus suggests that the protein contains a structure or function which confers this ability. A nucleolar localization signal, if its exists, could be dependent on or independent of a nuclear localization signal. A short polypeptide sequence conferring nucleolar localization has recently been identified in the $p27^{x-III}$ regulatory protein of human T-cell leukemia virus type I (39). If ICP27 possesses a similar signal, it must be located within the first 263 amino acid residues, since the 263R protein is localized to the nucleolus. If this is the case, however, it is not clear why the longer 406R protein does not accumulate efficiently in the nucleolus.

Transfection assays as probes of ICP27 function. The transfection assays described here provide a reasonable strategy for determining several properties of mutant ICP27 polypeptides (Table 3). Included in these are the ability of the protein to activate or repress gene expression, to localize into and within the cell nucleus, and to bind to a monoclonal antibody. In addition, it is possible to test whether mutant ICP27 molecules will complement the growth of an HSV-1 ICP27 null mutant. None of the truncated ICP27 polypeptides we tested, however, retained any ability to complement *d*27-1 growth, suggesting that each mutation has inactivated some essential function of ICP27. This is perhaps most surprising for the 504R mutant, which encodes a polypeptide in which only the last 8 of the 512 amino acids of ICP27 have been deleted and 4 novel amino acids have been inserted in their place. It is worth noting in this regard that the carboxyl-terminal portion of ICP27 is the region of the protein most highly conserved among the homologous herpesvirus proteins (5, 29).

The transfection assays are limited in two important respects with regard to information they can provide about ICP27 function. First, only a small percentage of cells are successfully transfected, making it difficult to perform biochemical or molecular biological analyses which might delineate mechanisms by which ICP27 modulates gene expression. In this regard, although our working model is that ICP27 is a transcriptional regulatory protein, the data obtained from transfection experiments do not directly test this hypothesis. The second limitation is that the transfection assays cannot fully mimic the complex regulatory interactions that occur in HSV-1-infected cells. It is therefore vital

to extend the genetic analysis of ICP27 to the context of the viral genome. In this way, specific models for ICP27 action, such as transcriptional regulation, can be directly tested. Furthermore, the effects of defined ICP27 mutations can be evaluated in the complex setting of the infected cell. It would be of special interest to study the phenotypes of HSV-1 mutants lacking ICP27 function entirely or possessing only one or the other of the two regulatory activities which we have identified in transfected cells.

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