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The Repressing and Enhancing Functions of the Herpes Simplex Virus Regulatory Protein ICP27 Map to C-Terminal Regions and Are Required To Modulate Viral Gene Expression Very Early in Infection

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The phenotypic properties of ICP27 temperature-sensitive and deletion mutants and the results of transient expression assays have demonstrated that ICP27 has a modulatory effect on viral gene expression induced by ICPs 0 and 4. In order to identify the regions of the ICP27 molecule that are responsible for its enhancing and repressing activities, 10 nonsense and 3 in-frame deletion mutations were introduced into the coding sequence of the cloned ICP27 gene. These mutant genes were tested in transient expression assays for their ability to complement an ICP27 null mutant and to enhance and repress expression from a spectrum of herpes simplex virus type 1 promoters in reporter CAT genes when expression was induced by ICP0 or ICP4. The results of assays with cloned mutant genes demonstrate that the ICP27 polypeptide contains two regions, located between amino acid residues 327 and 407 and residues 465 and 511, that contribute to its repressing activity. The amino acid region located between the two repressing regions (residues 407 to 465) is able to interfere with ICP27 repressing activity. None of the mutant genes exhibited efficient enhancing activity for any of the herpes simplex type 1 promoters tested, demonstrating that amino acids comprising the carboxy-terminal half of the ICP27 molecule, including the terminal phenylalanine residue, are required for wild-type enhancement as well as for efficient complementation of an ICP27 null mutant. Phenotypic characterization of an in-frame deletion mutant, vd3, and a previously isolated null mutant, 5dl 1.2 (A. M. McCarthy, L. McMahan, and P. A. Schaffer, J. Virol. 63:18–27, 1989), demonstrated that ICP27 is required to induce the expression of all classes of viral genes very early in infection and confirmed the requirement for ICP27 later in infection (i) to repress early gene expression, (ii) to induce wild-type levels of delayed-early or γ1 gene expression, and (iii) to induce true late or γ2 gene expression. The vd3 mutant, which specifies an ICP27 peptide lacking the repressing region between residues 327 and 407, is able to (i) repress early gene expression, consistent with the repressing ability of the d3 mutation in transient expression assays, (ii) induce the synthesis of significant but reduced levels of delayed-early (γ1) proteins and no γ2 proteins (thus vd3 exhibits a late protein phenotype intermediate between that of the wild-type virus and 5dl 1.2), and (iii) confer altered electrophoretic mobility on ICP4, demonstrating a role for ICP27 in the posttranslational modification of this essential regulatory protein. Taken together, these observations support a model in which ICP27 performs its regulatory activities differentially over time and mediates these activities indirectly via interactions with, and modifications of, ICP4 and perhaps other viral and cellular proteins.

The genome of herpes simplex virus type 1 (HSV-1) has been completely sequenced (33–35, 42) and encodes 72 viral proteins. These proteins are divided into four major kinetic classes on the basis of requirements for their expression and the times of their maximum rates of synthesis: immediate early, early, delayed early (sometime referred to as "leaky late" or γ1 proteins), and true late (γ2) (5, 25, 27, reviewed in reference 56). Viral genes of all four kinetic classes are transcribed by the cellular RNA polymerase II (1, 6) and are expressed in a coordinate and sequential fashion during the course of productive infection (26). Immediate-early genes are the first to be expressed during the replicative cycle, followed by the early genes, whose expression requires functional immediate-early proteins (13, 26, 43). Finally, the delayed-early and true late genes are expressed. Maximum expression of these latter two classes of genes requires the activities of both immediate-early and early gene products as well as viral DNA synthesis (24, 38). The primary difference between delayed-early (γ1) and true late (γ2) genes is that γ1 gene expression does not require viral DNA synthesis (although DNA synthesis is required for maximum expression of γ1 genes), whereas expression of γ2 genes is stringently dependent on viral DNA synthesis (24, 38). Immediate-early genes encode the major HSV regulatory proteins, early genes code for proteins involved in viral DNA synthesis, and delayed-early and late genes specify virion structural proteins.

Immediate-early genes are expressed in the absence of prior viral protein synthesis (5). These genes are transcriptionally activated by a protein (VP16) present in incoming virus particles (2, 4). VP16, together with cellular factors including the octomer-binding protein, forms protein-DNA complexes with the consensus sequence TAATGARAT, located in the 5′ promoter-regulatory regions of the five immediate-early genes (4, 34, 42). The immediate-early genes encode infected-cell polypeptides (ICPs) 0, 4, 22, 27, and 47 (41). The locations of the genes encoding immediate-early proteins in the HSV-1 genome are indicated in Fig. 1

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(top line). All immediate-early proteins except ICP47 are nuclear phosphoproteins (57) and act to regulate their own synthesis as well as the synthesis of proteins of later kinetic classes (9, 10, 13, 19, 31, 38, 39, 44).

Insight into the functional roles of each of the immediate-early proteins in the regulation of viral gene expression came initially from studies of viral mutants. Characterization of temperature-sensitive (ts) and deletion mutants in the five immediate-early genes has shown that only ICP4 and ICP27 are absolutely essential for virus replication (9, 10, 13, 29, 32, 43, 47, 48, 50, 54). Studies of mutants have also shown that ICP4 is involved in the down regulation of its own expression as well as that of other immediate-early genes and is required throughout the viral replicative cycle to up regulate the expression of early, delayed-early, and late genes at the level of transcription (9, 10, 12, 13, 43). Studies of mutants in the gene encoding ICP27 have shown that this protein is required to repress expression of certain immediate-early and early genes and is absolutely required for the transcriptional activation of late genes, a function that is independent of early protein and viral DNA synthesis (32, 46, 47). Compared with the wild-type virus, ICP0 deletion mutants grow well and induce the synthesis of wild-type amounts of viral proteins at high multiplicities of infection but they grow poorly and induce reduced levels of viral proteins at low multiplicities (48, 54). Thus, ICP0 plays an important although nonessential role in productive infection. ICP22 appears to be involved in the activation of late gene expression and has been shown to be essential for efficient virus replication in some cell types but not in others (50; C. Bogard, unpublished observations). ICP47 is apparently not essential for viral growth, at least in cell culture (29).

Transient assays that measure the regulatory effects of individual immediate-early proteins on the expression of a reporter gene (chloramphenicol acetyltransferase [CAT]) controlled by various HSV promoters have confirmed the findings obtained with viral mutants. Thus, ICP4 was shown to activate CAT expression controlled by early and late viral promoters and to repress expression from its own promoter (i.e., ICP4 is autoregulatory) by binding to a specific hexanucleotide (ATCGTC) near the ICP4 transcriptional start site (10, 15, 19, 31, 39, 44). Although phenotypic analysis of ICP0 deletion mutants has shown that this protein is not absolutely required for viral gene expression during productive infection, transient expression assays have shown that (i) ICP0 is a potent transactivator of all four kinetic classes of HSV promoters, as well as a variety of cellular promoters, and (ii) the transactivating activity of ICP0 for these promoters is increased synergistically in the presence of ICP4 (15, 19, 31, 38, 39, 44, 51, 52). The mechanism of ICP0-mediated transactivation is unknown at present.

In contrast to the activities of ICP0 and ICP4, ICP27 alone has little regulatory activity for HSV promoters in transient expression assays (9, 15, 51). An exception to this rule is the observation by Rice and Knipe that ICP27 by itself can transactivate the delayed-early glycoprotein B (gB) promoter (45). Despite its limited regulatory activity alone, ICP27 can both repress and enhance expression from a variety of HSV-1 promoters whose expression is induced by ICP0 or ICP4 or both (16, 45, 51, 55). Thus, ICP27 acts as a modulator of the regulatory functions of ICP0 and ICP4. Available data have not established whether ICP27 mediates its modulatory effects by direct interaction with ICP0 and ICP4, indirectly through interaction with or modification of viral or cellular proteins, or by direct or indirect binding to DNA. Although less well studied, neither ICP22 nor ICP47, alone or in combination with other immediate-early proteins, has been shown to exert regulatory effects on viral gene expression in transient expression assays.

Thus, of the five HSV-1 immediate-early proteins, only ICP0, ICP4, and ICP27 have been demonstrated to exhibit significant *trans*-regulatory activity. Systematic mutational analyses of the coding sequences for ICP4 and ICP0 have identified specific regions of these proteins that are involved in their respective transcriptional regulatory and DNA-binding activities, sites of phosphorylation, and nuclear and intranuclear localization (3, 12, 17, 40). As noted above, ICP27 has at least two major regulatory functions. It acts both as a repressor of selected immediate-early and early gene expression and as an enhancer of late gene expression. Like ICP27, the immediate-early protein E1A of adenovirus acts both as a transcriptional repressor and activator of viral gene expression (37). Moreover, these activities are specified by different regions of the polypeptide (28).

In order to assign the functional activities of repression and enhancement to specific regions of the ICP27 molecule and to gain further insight into the significance of these activities in the cascade of viral gene expression, we have introduced nonsense and in-frame deletion mutations into the coding region of the cloned ICP27 gene. We have compared mutant ICP27 proteins with the wild-type protein for their ability to complement an ICP27 null mutant and to repress and enhance expression of reporter genes cooperatively with ICPs 0 and 4. We have also characterized a mutant virus, vd3, containing an in-frame deletion that has lost its ability to enhance but retained its ability to repress HSV-1 gene expression. The results of studies of mutant plasmids in transient assays have (i) confirmed the ability of ICP27 to *trans* repress and *trans* enhance expression from HSV-1 promoters activated by ICP0 and ICP4, (ii) localized two functional domains responsible for the repressing activity of ICP27 within the hydrophobic carboxy-terminal one-third of the ICP27 molecule, (iii) demonstrated a requirement for the carboxy half of the ICP27 molecule for enhancing activity, and (iv) shown that the integrity of the carboxy-terminal phenylalanine residue of ICP27 is required for both enhancing activity and efficient complementation of an ICP27 null mutant. Studies of the mutant virus vd3 have confirmed previous findings that ICP27 is responsible for a distinct shift in the mobility of ICP4 in sodium dodecyl sulfate (SDS) gels, implicating ICP27 in the posttranslational modification of ICP4. The ability of ICP27 to modify ICP4 was also shown to be distinct from its repressing activity. Finally, studies of vd3 have revealed that ICP27 performs its repressing and enhancing functions in a manner that is dependent upon the time course of viral infection. Thus, ICP27 functions as an enhancer of viral gene expression at a very early stage in infection and as repressor of early gene expression and an enhancer of late gene expression at later stages in the HSV-1 replication cycle.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (Vero; ATCC CCL81) and ICP27-expressing Vero cells (3-3 cells [32]) were propagated as previously described (47). The wild-type KOS strain of HSV-1, the KOS-derived ICP27 deletion mutant 5dl 1.2 (32), and a mutant, vd3, generated in these studies were propagated and assayed as described previously (49).

Plasmids. (i) Immediate-early effector plasmids. Effector plasmids containing the immediate-early genes for ICPs 0, 4,

and 27 of strain KOS were used in these studies. Plasmid pW3, which contains the ICP0 gene, was obtained from Wendy Sacks (Dana-Farber Cancer Institute, Boston, Mass.) (48), and pn11ND, which contains the gene for ICP4, was obtained from Neal DeLuca (Dana-Farber Cancer Institute) (11). (Please note that we have designated the ICP4-containing plasmid pn11, originally described by Neal DeLuca, as pn11ND to distinguish it from the ICP27-containing plasmid pn11 generated in these studies.) Plasmids pW3 and pn11ND have been shown to transactivate HSV-1 early genes in transient expression assays (11). Plasmid pKHX-BH, which contains the ICP27 gene, was provided by Stanley Person (Pennsylvania State University, University Park, Pa.).

(ii) CAT reporter plasmids. Chimeric CAT plasmids pICP4CAT (also know as pIE3CAT [45]), ptkCAT, pVP5 CAT, and pL42CAT, which contain the CAT gene under control of promoters for the genes encoding ICP4 (an immediate-early protein), thymidine kinase (an early protein), VP5 (a delayed-early or γ1 protein), and L42 (a true late or γ2 protein), respectively, were obtained from Neal DeLuca (10). pgBCAT, which contains the promoter of the g B gene (a delayed-early gene), was provided by Steven Rice (Harvard Medical School, Boston, Mass.) (45). All enzymes and restriction enzyme linkers used to construct ICP27 nonsense and deletion mutants were obtained from New England BioLabs, Inc. (Beverly, Mass.) and used as suggested by the manufacturer. The promoters inserted into all of these plasmids were derived from the KOS strain of HSV-1.

DNA isolation. Bacterial plasmid DNAs were isolated and purified as described previously (3). CsCl-banded KOS- and vd3-infected cell DNAs were isolated as described by DeLuca et al. (8, 48).

Transfection. For transient expression assays, transfection of Vero cells was carried out by the calcium phosphate precipitation procedure followed by glycerol shock, as previously described (3). The amounts and types of plasmid DNAs used in these tests are indicated in the text and figure legends. Contransfection of ICP27-expressing 3-3 cells with 2 μg of infectious KOS DNA and 4 μg of linearized pd3 mutant plasmid during efforts to generate the vd3 mutant virus was carried out as described previously for marker rescue (47).

Construction of ICP27 mutant plasmids. A series of 10 nonsense and 3 deletion mutations were introduced into coding sequences of the cloned ICP27 gene in plasmid pKHX-BH. The locations of HSV DNA sequences in pKHX-BH relative to the viral genome and the locations of the mutations in pKHX-BH are shown in Fig. 1. Nonsense mutations in the ICP27 gene were generated as described previously by inserting a synthetic oligonucleotide containing a *Hpa*I site and translational termination codons in all three reading frames

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5'GGCTAGTTAACTAGCC3'
     | HpaI |
3'CCGATCAATTGATCGG5'
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into five *Nae*I sites, four *Sma*I sites, and the unique *Sal*I, *Stu*I, *Nsi*I, *Eco*NI, and *Sac*I sites in the cloned ICP27 gene (Fig. 1) (11). Since the ICP27-containing plasmid pKHX-BH has no *Hpa*I cleavage sites, *Hpa*I digestion of plasmids containing the synthetic oligonucleotide results in linear molecules, demonstrating the presence of the oligonucleotide in these plasmids.

In-frame deletion mutations in the ICP27 coding sequence were constructed by restricting pKHX-BH with the indicated enzymes (Fig. 1), filling in or trimming the protruding ends, and adding appropriate synthetic linkers at the indicated sites. For example, the 5′ protruding end of plasmid pd1 was trimmed and the 3′ protruding ends of plasmids pd2 and pd3 were filled in with Klenow, and 12-base-pair (pd1), 8-base-pair (pd2), and 10-base-pair (pd3) *Bgl*II linkers were blunt end ligated to the respective plasmids. The ligated plasmids were then cleaved with excess *Bgl*II to ensure that each plasmid contained only one copy of the linker (pKHX-BH has no *Bgl*II cleavage sites). The cleaved plasmids were religated with T4 ligase. The locations of the *Hpa*I insertion mutations and of the limits of deletion mutations (Fig. 1) were confirmed by restriction enzyme analysis.

Complementation. Complementation of the ICP27 deletion mutant 5dl 1.2 by peptides expressed transiently from cloned, mutated ICP27 genes was conducted in a manner similar to that described by Sacks et al. (47), except that Vero cells were used instead of CV-1 cells and the following procedural modifications were employed: 0.5 μg (instead of 0.3 μg) of plasmid DNA and 4.5 μg (instead of 4.1 μg) of salmon testis DNA in 0.3 ml (instead of 0.2 ml) of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline was used to transfect ~2 × 10^5 cells per 35-mm dish. Mutant 5dl 1.2 is an ICP27 deletion mutant that fails to replicate in Vero cells and expresses no detectable ICP27 polypeptide (32). Transfected cells were infected with 5dl 1.2 24 h after glycerol treatment. After 18 h postinfection, infected cells were harvested and progeny virus was assayed on 3-3 cells to quantify total virus (i.e., virus generated by complementation as well as any recombinants that may have been generated) and on Vero cells to quantify any replication-competent recombinants. 5dl 1.2 DNA provides no sequence homology to the left of the *Bam*HI site in the ICP27-containing *Bam*HI-*Hpa*I insert in plasmid pKHX-BH (32). As expected, no replication-competent recombinants between 5dl 1.2 and any of the plasmids tested were detected.

CAT assays. The in vitro assay for CAT activity was performed by a modification (10) of the method of Gorman et al. (21).

Southern blot analysis. DNA restriction fragments separated by agarose gel electrophoresis were transferred to nitrocellulose and hybridized to labeled probes by the method of Southern (53). The probe used for Southern blots was the plasmid pKHX-BH (Fig. 1). The probe was labeled with [^{32}P]dCTP and -dGTP (Dupont, NEN Research Products, Boston, Mass.) by nick translation (30).

Analysis of infected-cell polypeptides. Infected Vero cells were labeled with [^{35}S]methionine (Dupont, NEN Research Products) as indicated in figure legends. Lysates of radioactively labeled infected-cell cultures were prepared and electrophoresed on SDS-polyacrylamide gels as described previously (48).

RESULTS

ICP27 mutant plasmids. Ten nonsense and three deletion mutations were introduced into the coding sequence of the cloned ICP27 gene in plasmid pKHX-BH (Fig. 1). The genes containing nonsense mutations specified ICP27 peptides composed of from 26 (pn2) to 511 (pn11) amino acid residues of the 512-residue wild-type protein. The synthetic linker used to generate nonsense mutations was also introduced into sequences downstream of the 3′ transcription termination site of the ICP27 gene. The resulting plasmid, pn14, specifies the wild-type ICP27 protein. The three deletion

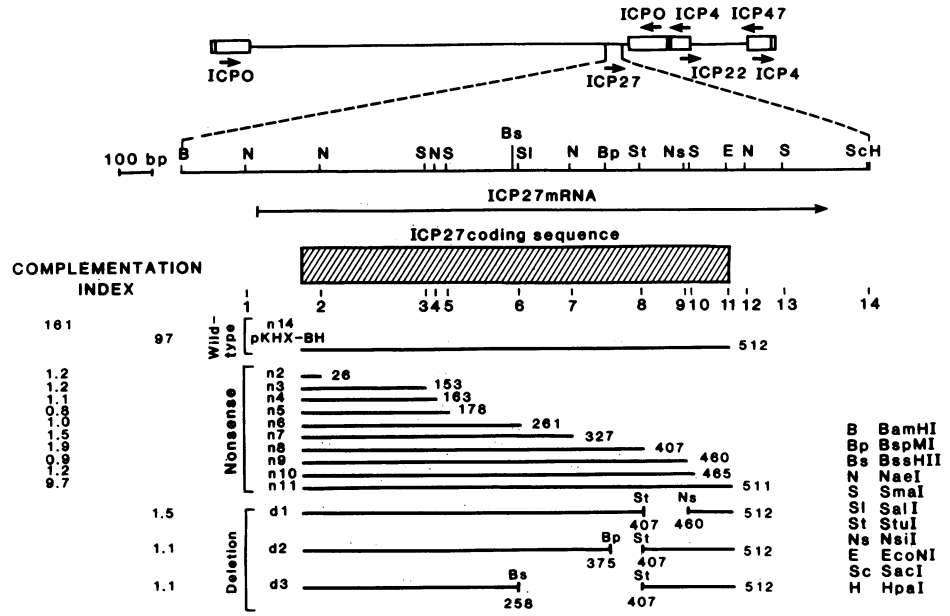

FIG. 1. Physical map of ICP27 nonsense (n) and deletion (d) mutations and complementation of 5dl 1.2 by ICP27 mutant plasmids. The locations of the immediate-early genes encoding ICPs 0, 4, 22, 27, and 47 are shown as arrows on the HSV-1 genome at the top of the figure. Below the genome is an expanded restriction map of the *Bam*HI-*Hpa*I viral DNA insert in plasmid pKHX-BH used to generate mutations in the ICP27 gene. Beneath the expanded map are shown the limits of the sequences specifying ICP27 mRNA, the direction of transcription of this mRNA, and the locations of the ICP27 coding sequence. The numbered vertical lines below the ICP27 coding sequence indicate the locations of nonsense mutations introduced into the ICP27 gene in pKHX-BH to yield the nonsense insertion mutant plasmids n1 through n14. The heavy lines in diagrams of nonsense mutants indicate the ICP27 amino acid sequence encoded by mutant plasmids. The heavy lines in deletion mutant diagrams indicate the DNA sequences that encode in-frame ICP27 amino acids. To the left of mutant diagrams are shown the complementation indices (CI) of mutant peptides for 5dl 1.2. (CI equals the ratio of the yield of 5dl 1.2 in Vero cells transfected with a mutant plasmid to the yield of 5dl 1.2 in Vero cells transfected with pBR322.)

mutations removed viral DNA sequences that encode 53 (pd1)-, 32 (pd2)-, and 149 (pd3)-amino-acid residues in the carboxy-terminal half of the ICP27 molecule.

Complementation of an ICP27 deletion mutant (5dl1.2) by mutant plasmids. In order to determine whether the mutated ICP27 genes specify peptides with ICP27-associated activities, mutant plasmids were first tested for their ability to supply these activities by complementation of the ICP27 deletion mutant 5dl 1.2 (32). As shown in Fig. 1 (far left), with the exception of the wild-type ICP27 protein, which produced complementation indices of 161 and 97 in two independent tests, peptides specified by nonsense mutant plasmids pn2 through pn10 and deletion mutant plasmids pd1, pd2, and pd3 failed to complement 5dl 1.2. Mutant plasmid pn11 specifies a peptide in which the carboxy-terminal amino acid residue, phenylalanine, was replaced by three amino acid residues (Leu, Ala, and Ser) following linker insertion. Plasmid pn11 was able to complement 5dl 1.2 to only 6% of the wild-type level (complementation index, 9.7). These results demonstrate that one or more essential ICP27-associated activities were affected by each of the mutations in our series of mutant plasmids and that replacement of the carboxy-terminal-most amino acid, phenylalanine, significantly reduced complementing activity for 5dl 1.2.

Repressing and enhancing activities of mutant plasmids. In order to identify regions of the ICP27 molecule that are required for its repressing and enhancing activities, mutant plasmids were tested for their ability to modulate the up regulatory effects of ICP0 or ICP4 on CAT reporter genes driven by various HSV-1 promoters. Reporter plasmids tested included ptkCAT, pgBCAT, pICP4CAT, pVP5CAT, and pL42CAT. The mutant effector plasmids tested were pn2, pn4, pn6, pn7, pn8, pn9, pn10, pn11, pn14, pd1, pd2, and pd3. pKHX-BH, which contains the wild-type ICP27 gene, and pn14 were used as positive controls. Plasmid pn14 contains a nonsense insertion mutation at the *Sac*I site outside the ICP27 coding sequence and, therefore, like pKHX-BH, specifies the wild-type ICP27 protein (see *Sac*I site downstream of the transcription termination site, Fig. 1).

(i) Repression of ptkCAT activity. Results of cotransfections of ptkCAT together with the various ICP27 alleles and plasmids expressing either ICP0 (pW3) or ICP4 (pn11ND) are shown in Fig. 2A. Consistent with previous findings (10, 19, 31, 38, 44), both ICP0 and ICP4 induced ptkCAT activity significantly above (~70-fold) the uninduced level, whereas wild-type ICP27 (pKHX-BH) exhibited no significant enhancing activity for the thymidine kinase (tk) promoter. Also consistent with previous findings was the ability of wild-type ICP27 (specified by pn14) to repress ptkCAT activity induced by ICP0 or ICP4 (51). The pn8 and pn11 forms of ICP27 also exhibited significant repression, but repression by pn8 was less efficient than that observed with the wild-type protein. The ICP27 peptides encoded by pn6 and pn7 exhibited modest repression of both ICP0- and ICP4-induced ptkCAT activity, whereas neither the pn2 nor the pn4 form of ICP27 exhibited repression of either ICP0- or ICP4-induced activity. Interestingly, the pn9 and pn10 forms of ICP27 reproducibly exhibited no repression of ICP4-induced activity, but they repressed ~30 and ~40% of ICP0-induced ptkCAT activity, respectively.

These observations indicate that the 80 amino acids present in the pn8 protein but absent from the pn7 protein (Fig. 1) specify significant repressing activity for the tk

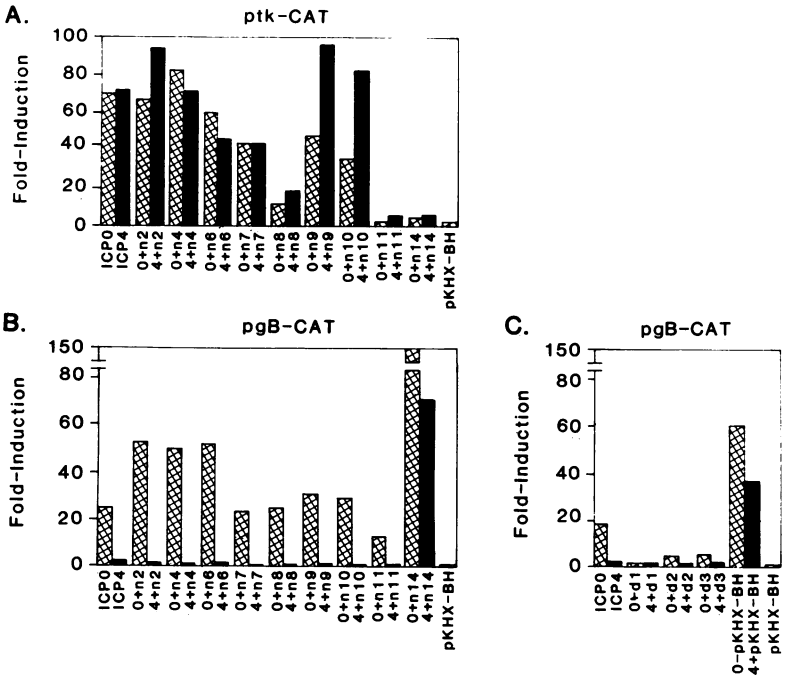

FIG. 2. Effect of wild-type and mutant ICP27 peptides on tkCAT and gBCAT expression induced by ICP0 or ICP4. Monolayers of Vero cells were cotransfected with 2 μg of ptkCAT or 4 μg of the pgBCAT reporter plasmids and with 2 μg of pBR322, 3 μg of either pW3 (encoding ICP0) or pn11ND (encoding ICP4), 2 μg of the indicated ICP27 mutant plasmid, or 2 μg of pKHX-BH or pn14. (Note: The pn11ND plasmid encoding ICP4 is distinct from the pn11 plasmid encoding the ICP27 gene mutated in the C-terminal phenylaline residue.) All transfections were brought up to 25 μg with salmon testis DNA. Transfected cells were harvested 2 days posttransfection, and cell extracts were assayed for CAT activity. The uninduced level of ptkCAT or pgBCAT activity was determined by cotransfection with pBR322 alone with no effector plasmid added (not shown). This uninduced level was arbitrarily set at 1.0. The "fold" induction of CAT activity in cotransfections with effector plasmids was calculated relative to the uninduced value.

promoter. Notably, addition to the pn8 peptide of 53 (pn9) and 58 (pn10) amino acids interfered with the ability of the pn8 peptide to repress. Likewise, the addition of 46 amino acids to the pn10 peptide to produce the pn11 peptide relieved the interference with repression exhibited by pn10 such that the pn11 peptide exhibited wild-type repressing activity. Thus, studies with nonsense mutations have identified an 80-amino-acid repressing region (327 to 407), a 58-amino-acid region (407 to 465) that interferes with the repressing activity of the 80-residue region, and a 46-amino-acid repressing region (465 to 511). Results with pn11 also demonstrate that insertion of the synthetic nonsense linker into the codon specifying the carboxy-terminal phenylalanine residue did not affect the ability of ICP27 to repress ICP0- or ICP4-induced activity.

Deletion plasmids pd1, pd2, and pd3 all specify the 46-amino-acid repressing region of ICP27 (Fig. 1). The pd1 plasmid also specifies the 80-amino-acid repressing region. By contrast, pd2 lacks 48 (357 to 407) of these 80 (327 to 407) amino acids and pd3 lacks all 80 residues. Notably, the protein specified by pd1 was more efficient than wild-type ICP27 in repressing ICP0-induced ptkCAT activity, whereas the pd2 protein was as efficient as the wild-type protein and pd3 was less efficient than wild-type ICP27 in this regard (see Fig. 4, right-hand panel). The increased repressing activity of pd1 relative to wild-type ICP27 is, by definition, due to the absence of residues 407 to 460. Such an effect is not unexpected when one recalls that these residues interfered with the repressing activity of the pn8 protein. The absence of 48 residues of the 80-amino-acid repressing region in pd2 had no effect on the repressing activity of ICP27 (see Fig. 4, right-hand panel), implying that the activity associated with this region is likely specified by residues 327 to 375. Supporting this view, the pd3 protein, which lacks the entire 80-residue region, was significantly less efficient than wild-type ICP27 in repressing ICP0-induced ptkCAT activity. The residual repressing activity retained by the pd3 protein is likely due to the fact that it contains 45 of the 46 residues in the carboxy-terminal-most region that specifies ICP27 repressing activity. Taken together, these data suggest that repression of ICP0- and ICP4-induced ptkCAT activity involves residues 327 to 375 and 465 to 511.

(ii) Enhancement of pgBCAT activity. Previous studies have shown that ICP27 is able to further enhance expression of pgBCAT activity induced by either ICP0 or ICP4 (45). We therefore attempted to identify the ICP27 amino acid sequences responsible for this enhancement. The results of transient expression assays in which the gBCAT reporter gene was cotransfected with wild-type and mutant ICP27 plasmids and plasmids expressing ICP0 or ICP4 are shown in Fig. 2B and C. In these tests, the wild-type form of ICP27 alone (specified by pKHX-BH) had no enhancing effect on the gB promoter, whereas pgBCAT activity was induced 20- to 25-fold by ICP0. ICP4 alone exhibited minimal enhancing activity for the gB promoter under the conditions used in these tests. Although the wild-type form of ICP27 specified by pn14 enhanced both ICP0- and ICP4-induced pgBCAT activity significantly, none of the nonsense (Fig. 2B) or deletion (Fig. 2C) mutant forms of the protein enhanced ICP4-induced activity and only pn2, pn4, and pn6 enhanced ICP0-induced activity (~twofold above the control level). The failure of pn11 to enhance pgBCAT activity must be due

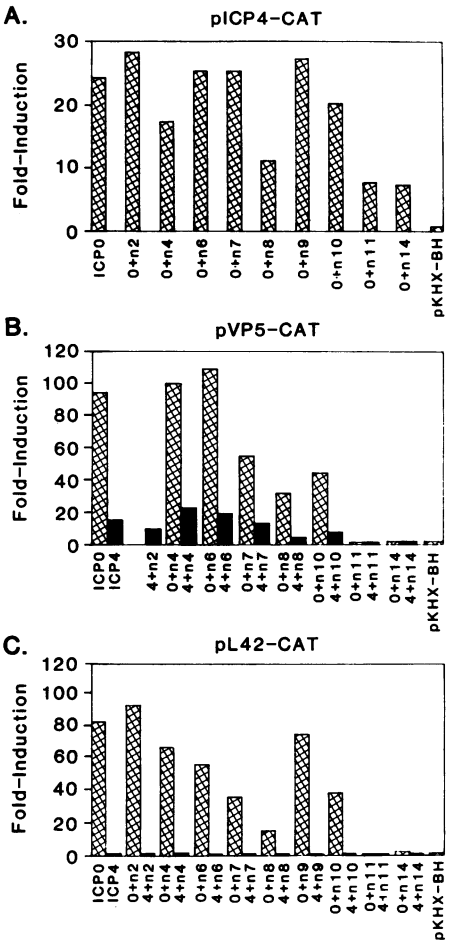

FIG. 3. Effect of wild-type and mutant ICP27 peptides on pICP4CAT, pVP5CAT, and pL42CAT expression induced by ICP0 or ICP4. For experiments with pICP4CAT, 2 μg of pICP4CAT was coprecipitated with either 1 μg of pBR322, 1 μg of pW3 (encoding ICP0), 1 μg of the indicated mutant ICP27 plasmid in the presence of 1 μg of pW3, or 1 μg of cloned pKHX-BH. Experiments with pVP5CAT and pL42CAT were performed and quantified in a manner identical to that described for ptkCAT and pgBCAT in the legend to Fig. 2.

to either the absence of the terminal phenylalanine residue or to its replacement by three amino acids brought about by linker insertion.

Taken together, these observations suggest that the carboxy terminus of ICP27 is required for the efficient enhancement of ICP0- and ICP4-induced transactivation of the gB promoter.

(iii) Repression of pICP4CAT activity. ICP0 is able to induce expression from the ICP4 promoter (10, 39), and ICP27 is able to repress this activity (38). Similarly, in our hands ICP0 enhanced pICP4CAT activity by approximately 25-fold (Fig. 3A) and the wild-type ICP27 protein specified by pn14 as well as the mutant peptide specified by pn11 repressed this activity with nearly equal efficiency. Significant repressive activity was also seen with pn8. The other mutant forms of ICP27 (pn2, pn4, pn6, pn7, pn9, and pn10) exhibited no significant repression of ICP0-induced pICP4CAT activity. These results are similar to those described above for repression of the tk promoter and support the existence of an 80-amino-acid region between residues 327 (pn7) and 407 (pn8) and a 46-amino-acid region between residues 465 (pn10) and 511 (pn11) that specify repressing activity.

(iv) Repression of pVP5CAT and pL42CAT activity. In order to identify the region(s) of the ICP27 peptide responsible for the modulatory effects of ICP27 on the expression of representative γ1 and γ2 genes (51), mutant peptides were tested for their ability to affect the expression of a γ1 (pVP5CAT) and a γ2 (pL42CAT) reporter gene.

In these studies, ICP27 alone (pKHX-BH) exhibited no enhancing or repressing activity for either gene, ICP0 exhibited a strong enhancing effect on the expression of both reporter genes, and ICP4 exhibited modest enhancing activity for pVP5CAT but not for pL42CAT (Fig. 3B and C). Wild-type ICP27, specified by pn14, and the pn11 mutant peptide repressed the enhancing activities induced by both ICP0 (for pVP5CAT and pL42CAT) and ICP4 (for pVP5CAT) to uninduced control levels. The ability to repress ICP0- and ICP4-induced activation of pVP5CAT was also a property of the pn8 mutant peptide and to a lesser extent of the peptides specified by pn7 and pn10 (for ICP0-induced activity). The relative strengths of the repressing activities of the various ICP27 mutant peptides for ICP0-induced pL42CAT activity was nearly identical to that seen with ptkCAT and pICP4CAT.

(v) Verification of the repressing activities of mutant peptides. In order to determine definitively whether the repressing activity exhibited by wild-type plasmids pKHX-BH and pn14 and mutant plasmids pn8, pn11 (Fig. 2A), pd1, pd2, and pd3 is a consequence of the activities of the corresponding ICP27 peptides, additional tests were conducted with ptkCAT (Fig. 4). In these tests, effector plasmids were cleaved within ICP27 coding sequences (*Sal*I for pn14, pKHX-BH, pn8, pn11, pd1, and pd2; *Nsi*I for pd3) or in plasmid sequences with *Pvu*II, before cotransfection of cells with ptkCAT and pW3 (see Fig. 1 for relevant cleavage sites). Cotransfection of *Sal*I- or *Nsi*I-digested plasmids with the ICP0 plasmid did not result in repression of ICP0-induced ptkCAT activity (i.e., CAT activity similar to that produced in control cells transfected with the ICP0 plasmid alone was observed). Therefore, interruption of ICP27 coding sequences in the wild-type and mutant plasmids impaired the ability of the peptides they encode to repress ICP0-induced ptkCAT activity. In contrast, cotransfection of *Pvu*II-digested plasmids with the ICP0 plasmid resulted in repression of ICP0-induced ptkCAT activity (i.e., CAT signals were similar to those of the undigested plasmids). Thus, the repressing ability of the ICP27 protein specified by wild-type plasmids pKHX-BH and n14 and mutant plasmids pn8, pn11, pd1, pd2, and pd3 requires the expression of the ICP27 proteins they encode.

One may argue that the repressing activity of the mutant forms of ICP27 is not the residual activity of ICP27 but a *trans*-dominant negative phenotype converted from a positive activity. If this were the case, this *trans*-dominant negative activity should have a negative effect on the gB promoter, in contrast to wild-type ICP27, which displays an enhancing effect. We did not observe such a negative effect, in that mutants which lost the ability to activate pgBCAT (e.g., pn8) did not acquire the ability to repress pgBCAT (Fig. 2B). On the other hand, the mutants resembled wild-type ICP27 in that they repressed expression from the ICP4, tk, VP5, and L42 promoters. Therefore, the mutant phenotypes reflect the repressing activity of the wild-type ICP27 molecule and can be used legitimately to map the region involved in repression.

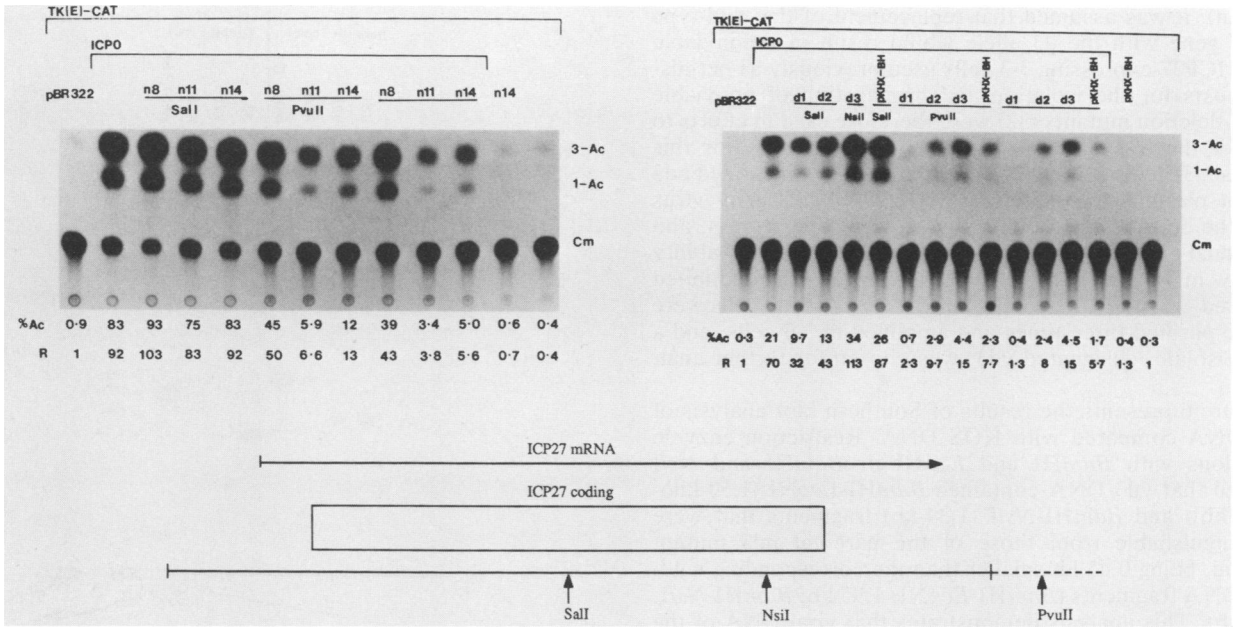

FIG. 4. Effect of intra- and extragenic cleavage of ICP27 mutant plasmids on ptkCAT expression induced by ICP0. Prior to transfection, plasmids either were cleaved with *Sal*I, *Nsi*I, or *Pvu*II or were not cleaved. Shown below the autoradiogram is the ICP27 coding sequence and the locations of the relevant restriction enzyme cleavage sites. This experiment was performed in a manner identical to that described in the legend to Fig. 2 for ptkCAT.

Collectively, the results of transient expression assays demonstrate that ICP27, which alone had little or no effect on the expression of any of the HSV-1 reporter genes tested, acts as a repressor of ICP0- and/or ICP4-induced expression of pICP4CAT, ptkCAT, pVP5CAT, and pL42CAT and as an enhancer of pgBCAT activity induced by ICP0 or ICP4. The results of transient expression assays with the ICP27 mutant plasmids are summarized in Fig. 5. The following conclusions can be drawn from these studies: (i) two regions of the ICP27 molecule exhibit repressing activity—one region between amino acid residues 327 and 407 and a second between residues 465 and 511, (ii) residues 407 to 460 are able to interfere with the repressing activity specified by residues 327 to 407, (iii) many regions of the carboxy-terminal half of the ICP27 protein are critical for the enhancing effect of ICP27 on the gB promoter and for efficient complementation of an ICP27 deletion mutant, 5dl 1.2, and (iv) substitution of a nonsense codon specifying three amino acids for the carboxy-terminal phenylalanine codon destroyed the enhancing activity of ICP27 but did not affect its repressing activity.

Introduction of the d3 mutation into the viral genome. As shown in Fig. 4, the mutant peptides encoded by deletion plasmids pd1, pd2, and pd3 were able to repress ICP0-induced ptkCAT activity in transient expression assays. Of the three mutants, the repressing activity of pd1 was greater than that of the wild-type ICP27 protein and that of pd3 was somewhat less than that of wild-type ICP27 (Fig. 4). Because the d3 mutation exhibited a repressing phenotype midway between that of wild-type ICP27 and the null phenotype (Fig. 4, right-hand panel), the d3 mutation was transferred into the viral genome by marker transfer. Since the pd3 mutant plasmid failed to complement the ICP27 deletion mutant 5dl1.2 in transient complementation assays (Fig. 1, left

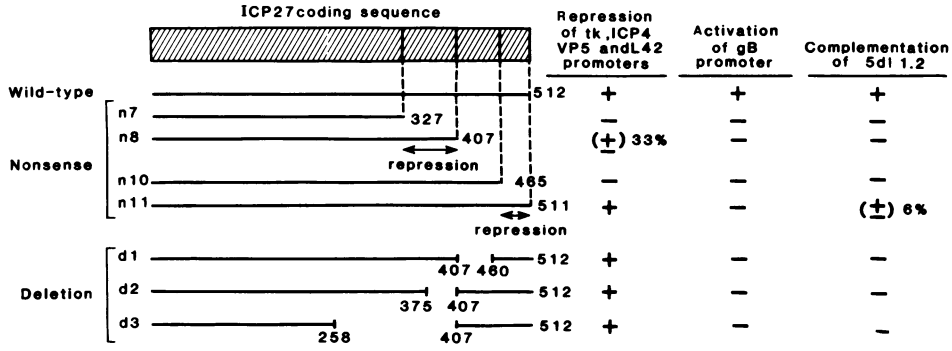

FIG. 5. Locations of the ICP27 primary amino acid sequences responsible for repression of CAT activity from reporter genes. Beneath the box representing the ICP27 coding sequence are shown the locations of the nonsense insertion (n) and deletion (d) mutations in mutant plasmids that define functionally important regions of the ICP27 molecule. The repressive and enhancing activities of mutant ICP27 plasmids are shown to the right of the figure.

column), it was assumed that replacement of the wild-type ICP27 gene with the d3 allele would result in a nonviable virus. ICP27-expressing 3-3 cells used previously as permissive hosts for the isolation and propagation of nonviable ICP27 deletion mutants (32) were therefore used in efforts to transfer the d3 mutation into the viral genome. For this purpose, 3-3 cells were cotransfected with the linearized pd3 mutant plasmid and infectious KOS DNA. Progeny virus from the cotransfected culture was plated on 3-3 cells, and individual plaques were picked and screened for their ability to grow in 3-3 and Vero cells. Plaque isolates that exhibited impaired growth in Vero cells but grew well in 3-3 cells were plaque purified three times and amplified in 3-3 cells, and a single isolate, designated vd3, was chosen for further analysis.

Figure 6 presents the results of Southern blot analysis of vd3 DNA compared with KOS DNA. Restriction enzyme digestions with *Bam*HI and *Eco*NI or *Bam*HI and *Nsi*I showed that vd3 DNA contained *Bam*HI-*Eco*NI (1.50-kilobase [kb]) and *Bam*HI-*Nsi*I (1.34-kb) fragments that were indistinguishable from those of the parental pd3 mutant plasmid, being 0.45 kb smaller than the corresponding wild-type DNA fragments (*Bam*HI-*Eco*NI, 1.95 kb; *Bam*HI-*Nsi*I, 1.79 kb). This analysis demonstrates that viral DNA of the vd3 mutant contains the deletion present in the parental plasmid.

Phenotypic analysis of ICP27 deletion mutants vd3 and 5dl1.2. (i) Growth properties. The results of single-cycle growth experiments shown in Table 1 demonstrate the following points. (i) When grown and assayed in Vero cells, no infectious vd3 or 5dl1.2 was detected, reflecting the nonpermissiveness of Vero cells for these mutants. This is in contrast to KOS, which grew and plated well in Vero cells. (ii) When grown in Vero cells and assayed in 3-3 cells, both vd3 and 5dl 1.2 yielded low titers of virus. This virus was likely residual unadsorbed mutant virus, since the plaques were noticeably smaller than KOS plaques on 3-3 cell monolayers. (iii) The ICP27-transformed cell line, 3-3, complemented both mutants efficiently, as demonstrated by the titers and plating efficiencies of the mutants on these cells. (iv) When vd3 was grown in 3-3 cells and assayed on Vero cells, no detectable wild-type recombinants were observed. This demonstrates that although vd3 DNA contains sequences flanking the deletion that are homologous to the ICP27-containing fragment resident in 3-3 cells (32), the superinfecting mutant virus did not recombine detectably with resident ICP27 sequences.

(ii) Immediate-early polypeptide synthesis. Figure 7 (cyclo$^+$) shows the immediate-early polypeptide profiles of KOS-, 5dl 1.2- and vd3-infected Vero cells in the presence of dactinomycin following removal of cycloheximide. Although KOS-infected cells contained significant amounts of ICP27 (Fig. 7, arrow), 5dl 1.2 produced no detectable ICP27 polypeptide. This is consistent with the genotype of 5dl 1.2, which lacks the transcriptional start site as well as portions of the promoter and coding sequence of the ICP27 gene (32). As expected, vd3, which contains an in-frame deletion mutation in ICP27 coding sequences (Fig. 1), did not produce a band that migrated in the position of wild-type ICP27 but rather produced a prominent band of the size expected of the truncated vd3 ICP27 polypeptide.

In HSV-infected cells, ICP4 is often present as multiple electrophoretic forms, presumably as a consequence of different levels of phosphorylation of the ICP4 peptide (13, 41, 57). Recently, Rice and Knipe observed a change in the electrophoretic mobility of ICP4 in cells infected with an

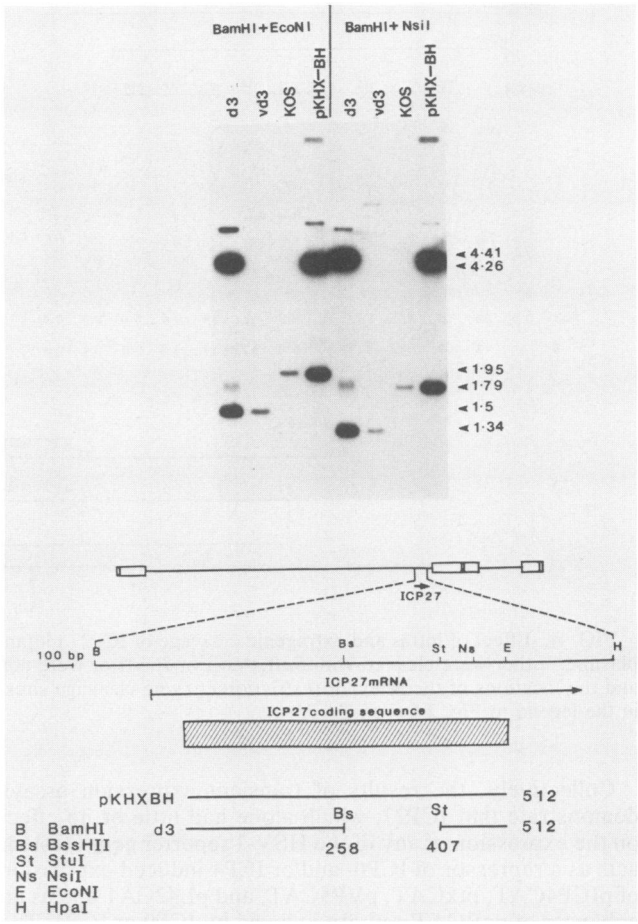

FIG. 6. Southern blot analysis of vd3 and KOS DNAs relative to the viral DNA inserts in plasmids pd3 and pKHX-BH. Viral (vd3 and KOS) and plasmid (pd3 and pKHX-BH) DNAs were digested with either *Bam*HI and *Eco*NI (left-hand lanes) or *Bam*HI and *Nsi*I (right-hand lanes). The digested DNAs were electrophoretically separated on a 0.7% agarose gel, transferred to nitrocellulose, and probed with ^{32}P-labeled pKHX-BH. Sizes of DNA fragments in kilobases are indicated on the right of the autoradiogram. Shown below the autoradiogram in descending order are a diagram of the KOS genome, an expanded diagram of the *Bam*HI-*Hpa*I viral DNA insert in plasmid pKHX-BH, the locations of relevant restriction sites, the location and direction of transcription of ICP27 mRNA, the location of ICP27 coding sequences, and the genotypes of the wild-type ICP27 gene in pKHX-BH and the d3 deletion mutation in plasmid pd3.

ICP27 ts mutant (45). This implies that ICP27 may be involved either directly or indirectly in the posttranslational modification of ICP4. In our hands, the ICP4 species synthesized in cells infected with 5dl 1.2 or vd3 migrated more slowly than the ICP4 species synthesized in KOS-infected cells. This difference was also seen in immunoprecipitation experiments in which monoclonal antibody to ICP4 was used (data not shown). Although the d3 mutation is able to repress viral gene expression as described above, it does not confer wild-type-like electrophoretic mobility on ICP4. This indicates that the repressive function associated with ICP27 is distinct from the function that affects ICP4 electrophoretic mobility. No significant alteration was detected in the quantity of ICP4 or in the quantities or electrophoretic mobilities of ICPs 0 or 22 in mutant infected cells.

TABLE 1. Growth of KOS, vd3, and 5dl 1.2 in Vero and ICP27-transformed cells

Virus	Virus yield (PFU/ml) when grown in[a]:			
	Vero cells		3-3 Cells	
	Vero	3-3	Vero	3-3
KOS	7.6×10^7	1.0×10^8	1.2×10^8	1.3×10^8
vd3	$<10^1$	5.4×10^3	$<10^{2b}$	5.1×10^7
5dl 1.2	$<10^1$	4.3×10^1	$<10^2$	7.6×10^7

[a] Monolayers of Vero or 3-3 cells were infected with the indicated virus at a multiplicity of infection of 5 PFU/cell. At 18 h postinfection, infected monolayers were harvested by scraping cells into medium. Suspensions were then frozen, thawed, sonicated, and clarified by low-speed centrifugation. Supernatant fluids were assayed on Vero monolayers.

[b] When vd3 and 5dl 1.2 were grown in permissive 3-3 cells and assayed on nonpermissive Vero cells, monlayers of the latter cells exhibited uniform nonspecific cytopathic effects at low dilutions; this was likely due to cell killing at high multiplicity infection.

(iii) Viral polypeptide synthesis. Previous studies of ICP27 ts and deletion mutants and the results of transient expression assays have suggested that ICP27 is required to down regulate early gene expression and to enhance late gene expression. To evaluate the effect of the d3 and 5dl1.2 mutations on the synthesis of early and late viral polypeptides, mutant and wild-type infected Vero cells were labeled from 5 to 17 h postinfection with [^{35}S]methionine (Fig. 7, cyclo$^-$).

The results of this experiment indicate that ICP27 is required to repress the expression of some early genes and to activate the expression of others. For example, cells infected with the ICP27 null mutant 5dl 1.2 overproduced early proteins (ICP6 and ICP8) but underproduced the early protein ICP41 relative to wild-type virus. Consistent with previous observations, 5dl 1.2 induced the synthesis of markedly reduced levels of proteins of the γ1 class (ICP5, -25, and -44) and no true late (γ2) proteins (ICP1/2, -15, -19/20, -35, and -43) (32). Like 5dl 1.2, vd3-infected cells underproduced ICP41 and failed to produce true late (γ2) proteins. The synthesis of pgB and gB relative to KOS-infected cells was not greatly affected in either 5dl 1.2- or vd3-infected cells in these experiments, and host protein synthesis was not efficiently shut off in cells infected with either mutant. Although vd3 is phenotypically similar to 5dl 1.2, the two mutants differ in two regards. vd3 induced the synthesis of early proteins (ICP6 and -8) and especially ICP6, at a level lower than that of 5dl 1.2 but slightly higher than that of wild-type virus, and it induced some (γ1) proteins (e.g., ICP5 and ICP25) at levels lower than that of wild-type virus but slightly higher than that of 5dl 1.2.

Three conclusions can be drawn with regard to the phenotype of vd3, based on the polypeptide profiles shown in Fig. 7 (cyclo$^-$). (i) The ability of vd3 to repress early gene expression to levels between those of 5dl 1.2 and wild-type virus correlates with the repressing ability of the pd3 mutant plasmid for the early target gene tkCAT in transfection assays (Fig. 4, right-hand panel). (ii) Unlike previously described ICP27 ts and deletion mutants (32, 47), vd3 induces the synthesis of significant levels of (γ1) proteins (i.e., ICP5 and ICP25). (iii) Like the ICP27 ts and deletion mutants (32, 47), vd3 is unable to up regulate true late (γ2) gene expression and is defective in the shutoff of host protein synthesis.

In order to establish that the viral polypeptide phenotype of vd3 is a consequence of the absence of ICP27 and not the effect of mutations in other regions of the genome, the mutation in vd3 was rescued by standard procedures (47) by using the *Bam*HI-*Hpa*I DNA fragment in plasmid pKHX-BH (Fig. 1). The rescued virus, vd3R, exhibited wild-type polypeptide profiles in both cycloheximide reversal and

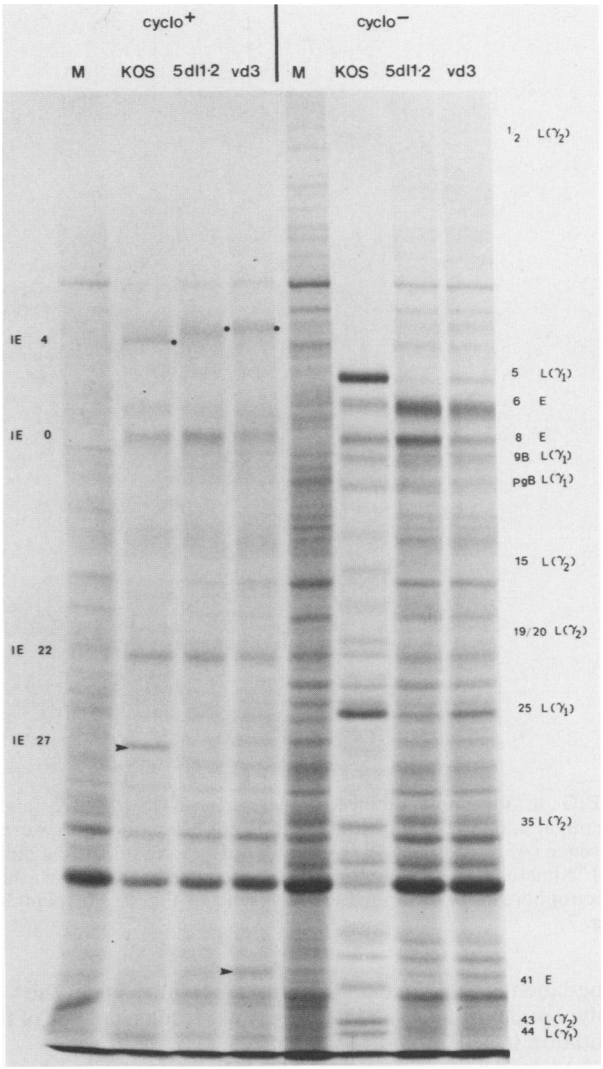

FIG. 7. Polypeptide profiles of KOS-, 5dl1.2-, and vd3-infected cells. Left-hand lanes (cyclo$^+$); Vero cell monolayers were preincubated for 1 h at 37°C in the presence of 75 μg of cycloheximide per ml. They were then infected with 10 PFU of the indicated viruses per cell in the presence of the same concentration of cycloheximide. At 6 h postinfection, cycloheximide-containing medium was removed and monolayers were washed and overlaid with medium containing 10 μg of dactinomycin per ml and 40 μCi of [^{35}S] methionine per 35-mm plate. After a further 3 h of incubation, infected cells were harvested and analyzed by SDS-polyacrylamide gel electrophoresis. Right-hand lanes (cyclo$^-$); Vero cell monolayers were infected with 10 PFU per cell at 37°C, labeled with the same concentration of [^{35}S]methionine indicated above from 5 to 17 h postinfection, and processed for SDS-polyacrylamide gel electrophoresis analysis. The SDS polypeptides were electrophoretically separated in 7% polyacrylamide gels. Selected immediate-early infected cell polypeptides (ICP4, -0, -22, and -27) are indicated in the left-hand margin. Selected early (ICP6, -8, and -41); delayed-early (γ1: ICP5, gB, pgB, -25, and -44); and true late (γ2: ICP1/2, -15, -19/20, -35, and -43) polypeptide bands are indicated on the right-hand side of the figure.

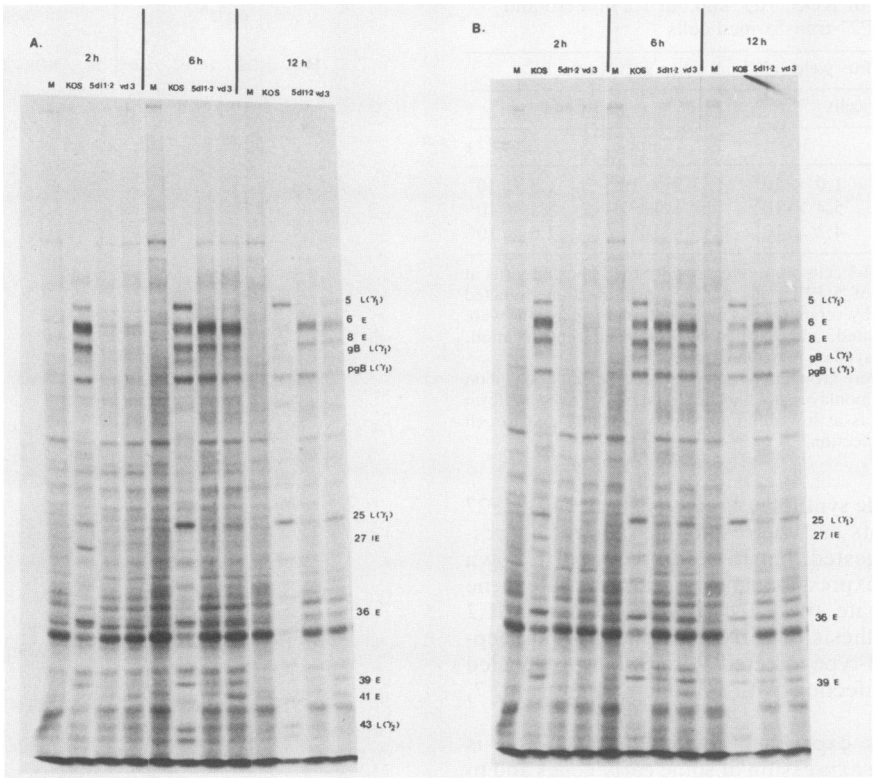

FIG. 8. Viral polypeptides synthesized in KOS-, 5dl 1.2-, and vd3-infected Vero cells in the absence (A) and presence (B) of PAA (400 μg/ml) at 2, 6, and 12 h postinfection. Vero cell monolayers were infected with 10 PFU of the indicated viruses and incubated at 37°C in the absence (A) or presence (B) of 400 μg of PAA per ml. At the indicated times postinfection, cells were pulse-labeled for 30 min with 100 μCi of [^{35}S]methionine per 35-mm plate. Monolayers were then washed, and cell lysates were prepared and analyzed by SDS-polyacrylamide gel electrophoresis as described in the legend to Fig. 7. Polypeptide designations on the right are described in greater detail in the legend to Fig. 7.

long-labeling experiments (data not shown). Hence, the vd3 polypeptide profiles shown in Fig. 7 are a consequence of the mutation in ICP27 coding sequences.

(iv) Kinetics of viral protein synthesis. To ascertain whether the alterations in gene expression observed in vd3-infected cells (Fig. 7) are due to the effect of reduced viral DNA synthesis and to examine the rates of polypeptide synthesis in KOS-, 5dl 1.2-, and vd3-infected cells, KOS- and mutant-infected Vero cells were pulse-labeled for 30 min at 2, 6, and 12 h postinfection with [^{35}S]methionine in the presence and absence of the DNA synthesis inhibitor phosphonoacetic acid (PAA).

The protein profiles shown in Fig. 8A (PAA^-) and B (PAA^+) demonstrate the following. (i) At 2 h postinfection, both vd3 and 5dl1.2 failed to induce the synthesis of detectable viral proteins, whereas viral proteins of all classes were detected in KOS-infected cells (Fig. 8A and B). This observation demonstrates that ICP27 is required to enhance the expression of all viral genes at a very early stage in virus replication. Because both mutants contain wild-type copies of ICPs 0 and 4, the major activators of viral gene expression, the absence of the activating functions of these two proteins at 2 h postinfection implies that ICP27 has an effect on one or both of these proteins. (ii) At 6 h postinfection, both vd3 and 5dl 1.2 induced levels of early gene expression equivalent to those of wild-type virus, whereas γ1 gene expression was markedly reduced relative to KOS. These observations indicate that early gene expression, although slow to be initiated in mutant-infected cells, reached wild-type levels by 6 h postinfection. This was not the case for γ1 proteins. (iii) At 12 h postinfection, the polypeptide profiles of the mutants had changed little from the 6-h profile, whereas in KOS-infected cells, early gene expression was repressed and γ1 gene expression was considerably augmented. (iv) The mutant profiles were not significantly different in the presence and absence of PAA, whereas early gene expression was repressed more efficiently in the absence than in the presence of PAA, consistent with previous findings (32). (v) As in the case of long-labeling experiments, the major difference in the phenotypes of vd3 and 5dl 1.2 was manifest as a slightly greater repression of early gene expression by vd3 than by 5dl 1.2. This difference was most notable at 12 h postinfection.

DISCUSSION

ICP27 is a multifunctional regulatory protein that possesses at least two distinct activities, namely repression and enhancement of ICP0- and ICP4-induced gene expression. Although the mechanism by which ICP27 mediates its regulatory effects is not clear, the observation that ICP27-stimulated viral gene expression occurs concomitantly with increased rates of the transcription strongly suggests that it exerts its up regulatory effect at the level of transcription (16, 32). The ICP27 protein is a nuclear phosphoprotein composed of 512 amino acids, with an apparent molecular mass of 63 kilodaltons (41). Like ICP4, phosphate has been shown to cycle on and off the nascent ICP27 protein during infec-

tion (57). Although ICP27 has been reported to bind to DNA (23), it is not known whether this binding is specific or nonspecific or whether it involves interaction with other viral or cellular proteins. The study described herein was designed to begin to relate the specific structural features of the ICP27 molecule with its repressing and enhancing activities.

Functional regions of the ICP27 protein. Although the requirement for the hydrophilic amino portion of ICP27 was not specifically assessed in these studies, Hardwicke et al. have shown that it is not essential for either the repressing or enhancing activity of the protein (22). The importance of the carboxyl portion of the ICP27 molecule to its essential regulatory functions is suggested by the fact that the mutations in all ICP27 ts mutants mapped to date are located in this region (47) and by the existence of extensive amino acid sequence conservation between this region of ICP27 and the carboxy-terminal halves of the related varicella-zoster virus gene 4 product, the Epstein-Barr virus BMLF1 product, and the IE 52k gene product of herpesvirus saimiri (7, 20; D. J. McGeoch, V. G. Preston, S. K. Weller, and P. A. Schaffer, *in* S. J. O'Brien, ed., *Genetic Maps*, in press).

In an effort to identify regions of the ICP27 primary amino acid sequence required for its repressing and enhancing functions, a series of nonsense and in-frame deletion mutations were introduced into the ICP27 coding sequence. Because they can be used to delete defined stretches of amino acids, these two types of mutation are especially useful in assessing the activities specified by individual regions of a protein and in identifying functional domains prior to more detailed analysis by amino acid deletion and substitution mutagenesis.

(i) Repression. The results of transient expression assays with mutant plasmids demonstrated that two regions of the ICP27 primary amino acid sequence specify repressing activity for immediate-early, early, and late promoters: an 80-amino-acid region between residues 327 and 407 and a 46-amino-acid region between residues 465 and 511. The 80-amino-acid region is not absolutely required for the repressing activity of ICP27, but it is necessary for wild-type levels of repression, since the pd3 peptide which lacks this region was able to repress ICP0- or ICP4-induced expression from the early tk promoter significantly.

The pn11 peptide but not the pn10 peptide efficiently represses ICP0- or ICP4-induced expression of immediate-early, early, and late promoters. This suggests that the 46-amino-acid region between residues 465 (pn10) and 511 (pn11) also contributes to the total repressing activity of ICP27. Amino acid sequence analysis has shown that the 46-residue region contains a putative zinc finger motif as well as four serine and two threonine residues which may serve as phosphorylation sites. Similar motifs are present in other transcription proteins.

Lying between the 80- and 46-amino-acid repressing regions of ICP27 is a 58-amino-acid sequence (407 to 465) that is not required for repressing activity but is able to interfere with the repression specified by the 80-residue sequence. Analysis of the primary and secondary structure of ICP27 reveals that the 58-amino-acid region between amino acids 407 (pn8) and 465 (pn10) is the most hydrophobic and hence the most rigid portion of the molecule. It is possible that the additional amino acid residues specified by pn9 and pn10 but not by pn8 in the absence of the remaining carboxy terminus (amino acids 465 to 512) may cause the pn9 and pn10 peptides to assume a conformation that masks the activity of the 80-amino-acid repressing region. As in the case of the mutant peptide specified by pd1 (Fig. 4, right-hand panel), mutations in the 58-amino-acid region would likely yield peptides with greater repressing activity than the wild-type protein. Such mutants might exhibit a *trans*-dominant phenotype (i.e., the ability to inhibit viral growth) even in the presence of wild-type ICP27.

(ii) Enhancement and complementation. None of the ICP27 mutant peptides tested in these studies was able to enhance ICP0- or ICP4-induced pgBCAT expression. Thus, specific amino acid sequences lying between residues 258 and 407 (pd3), 375 and 407 (pd2), 407 and 460 (pd1), and 460 and 512 (pn9) were shown to be required for pgBCAT enhancing activity. Of special interest was the observation that pn11 peptide, which contains 511 of the 512 residues in ICP27 (and hence contains the sequences deleted in mutant peptides specified by pd1, pd2, and pd3), was also unable to enhance gB promoter activity or to complement an ICP27 null mutant efficiently. These observations have led us to conclude that unlike its repressing function, which appears to be specified by at least two discrete domains of the ICP27 protein, the enhancing and complementing functions of ICP27 involve a larger portion of the carboxy half of the ICP27 molecule. Consequently, enhancing activity would be particularly sensitive to mutations that produce alterations in the conformation of the protein.

Mutant peptides were tested in this study for their ability to modulate ICP0- and ICP4-induced gene expression from five viral promoters representing genes of the four major kinetic classes. A comparison of the relative repressing activities of mutant peptides for the tk, ICP4, VP5, and L42 promoters induced by ICP0 revealed a strikingly similar pattern (compare Fig. 1A and Fig. 2B, C, and D). These relative activities were also evident in the repression of ptkCAT activity induced by ICP4. Together, these observations suggest a common mechanism underlying the repression of both ICP0- and ICP4-induced promoter activity by ICP27. They further suggest that the mechanism of repression is the same for all four classes of promoter.

The results of the transient expression assays conducted in this study confirm and extend the results of other investigators. Thus, the 80-amino-acid region able to repress ICP0- or ICP4-induced expression from four classes of promoter identified in this study is a subset of the 143-residue repressing region (263 to 406) described by Rice et al. for the early ICP8 promoter (46). Similarly, the 46-amino-acid repressing region shown for the same four classes of promoter in this study is a subset of the 78-residue repressing region (434 to 512) described by Hardwicke et al. for the early tk promoter (22). Together, these three studies suggest that repression of ICP0- or ICP4-induced gene expression is specified by at least two regions of the ICP27 molecule.

Our studies agree well with those of Hardwicke et al. concerning the region of the protein required for enhancement of ICP0- or ICP4-induced gene expression in that most of all of the carboxyl half of the protein is required for this activity (22). These results differ from those of Rice et al., in which a peptide specifying only the amino terminal 263 amino acids exhibited partial enhancing activity (46). The reasons for this discrepancy are currently unclear.

Complexities of transient expression assays. Investigators in several laboratories have clearly demonstrated that ICP27 is capable of both enhancing and repressing ICP0- or ICP4-induced viral gene expression in transient expression assays. Despite the consensus with regard to the activities of ICP27, marked discrepancies in the results of tests with individual viral promoters are well documented. Thus, for example,

Sekulovich et al. (51) observed inhibition of gCCAT activity by ICP27 in the presence of ICPs 0 and 4, whereas Rice et al. (46) noted an ICP27-specific enhancement of ICP4-induced gCCAT activity, and Shapira et al. observed no effect of ICP27 on gCCAT activity induced by either ICP0 or ICP4 (52). Additionally, ICP0- or ICP4-induced VP5CAT activity was repressed in the presence of ICP27 in our hands but was further stimulated in the studies of Sekulovich et al. (51) and Hardwicke et al. (22); inhibition of ICP0- or ICP4-induced expression of gBCAT was observed by Sekulovich et al. (51), whereas a further stimulation of ICP0- or ICP4-induced expression of gBCAT was observed by Rice et al. (46) and in the present study.

The molecular basis for these inconsistencies is not yet clear; however, factors that are likely to affect the qualitative and quantitative results of transient expression assays with ICP27 include the following. (i) Sequence content of promoters in both inducer and reporter plasmids is critical (18). In this regard, it is essential that all relevant *cis*-acting elements and other factors determining transcript processing and messenger efficiency be included in the promoters of both kinds of plasmid. It is possible that we are as yet unaware of the existence of all elements that affect gene expression in transient assays. (ii) The relative concentrations of inducer and reporter plasmids may affect the quantitative results of transient assays. Here, the promoters of both reporter and inducer plasmids may compete for critical cellular factors, as indicated by the findings of Rice et al. (46). (iii) Cell type and metabolic state of cells are also important. As demonstrated by Everett, the efficiency of gene expression in transient expression assays is affected both by cell type and the metabolic state of the cell (18). It is possible that changes in the abundance of cellular factors may produce the quantitative changes observed in ICP27-associated activities, whereas structural modification of these factors may produce qualitative changes.

Indeed, it is likely that all three of the above-mentioned factors play some role in determining the outcome of transient expression assays. Given the complexity of the environment in the infected cell, transient expression assays continue to offer advantages for evaluating the roles of individual proteins in regulating viral gene expression. Any conclusions reached from these studies, however, must ultimately be confirmed in infected cells, because transient assays tell us only what can occur and not what actually does occur in the presence of the full complement of viral gene products and virus-induced cellular factors. We are therefore in the process of transferring our ICP27 mutations into the background of the viral genome.

Requirements for ICP27 during virus infection. (i) ICP27 is required to induce wild-type levels of expression of all classes of viral genes early in infection. The strong up regulatory activity of ICPs 0 and 4 for all classes of viral genes has long been recognized. Despite the presence of wild-type genes for ICP0 and ICP4 in the genomes of vd3 and 5dl 1.2, these mutants were unable to induce detectable levels of viral proteins at very early times postinfection (2 h), demonstrating a requirement for ICP27 very early in the viral replicative cycle. The requirement for ICP27 is not absolute, however, in that wild-type levels of certain early genes were detected in mutant-infected cells by 6 h postinfection.

(ii) ICP27 is required to repress the expression of early genes and cellular genes early in the replication cycle. The requirement for ICP27 in the repression of early gene expression is clear from several studies of viral protein synthesis in ts and deletion mutant-infected cells (32, 46, 47). In all cases, early proteins were markedly overproduced. This requirement for ICP27 is well illustrated by the subtle differences in early gene expression seen in 5dl 1.2- and vd3-infected cells. In cells infected with 5dl 1.2, a null mutant which lacks all ICP27-associated activities, overexpression of early genes was maximal (see for example, Fig. 7, cyclo$^-$), whereas in cells infected with vd3, which retains some ICP27-associated repressing activity in in vitro assays, early gene expression was repressed to a significant extent. Repression may occur either through ICP27 and its interactions with other regulatory proteins or through the agency of late proteins whose expression is dependent upon ICP27. Should mutants that retain activating activity for late genes and lose repressing activity for early genes be isolated, the latter mechanism would be verified.

(iii) ICP27 is required to induce wild-type levels of delayed-early and late gene expression throughout the replication cycle. Although the expression of selected early genes reached wild-type levels midway in the replicative cycle in these studies, the expression of delayed-early (γ1) proteins was significantly reduced in mutant-infected cells relative to that seen in wild-type-infected cells, even at late times in the replicative cycle. A stringent requirement for ICP27 in true late (γ2) gene expression is clear in that proteins of this class have not yet been detected in ICP27 deletion mutant-infected cells (46, 47; this study).

Thus, the enhancing activity of ICP27 on ICP0- and ICP4-induced gene expression is required throughout the viral replicative cycle, early in infection for all classes of viral genes and at later times for delayed-early and late genes.

Whether the ICP27-associated activity required to down regulate ICP0- and ICP4-induced early gene expression is the same as the ICP27-associated activity required to shut off cellular protein synthesis remains to be determined. With regard to the effect of ICP27 on cellular protein synthesis, it should be noted that although ICP27 is required to down regulate cellular protein synthesis in general, the enhanced synthesis of at least one cellular protein, p40, is dependent upon the expression of ICP27 (14).

In view of the multiple activities of ICP27 and the differences in the regions of the protein that specify them, it is not surprising that available ICP27 mutants exhibit differing levels of these activities, as observed in long-labeling experiments. For instance, mutants 5dl 1.2 and tsY46 overproduce early proteins (e.g., ICP6 and ICP8) and are the most restrictive of all mutants examined to date in the production of delayed-early (γ1) proteins (e.g., ICP5 and ICP25) (32, 47). Mutants tsE5 and tsE6 overproduce early proteins most efficiently and are least restrictive in the induction of γ1 protein synthesis (47). vd3 induces the synthesis of intermediate levels of both early and delayed-early proteins (this study). Notably, none of the mutants induces detectable levels of γ2 proteins and none is able to repress host cell protein synthesis.

Given the complexities of the enhancing and repressing activities of ICP27, long-labeling experiments are less informative than pulse-labeling experiments in assessing the effects of mutations on protein synthesis. Fine mapping of the individual regions of the ICP27 molecule responsible for its repressing and enhancing functions together with pulse-labeling experiments in which mutant viruses are used should ultimately succeed in correlating individual amino acids with the various effects of ICP27 on viral and cellular protein synthesis.

Mechanisms of enhancement and repression of ICP0- and

ICP4-induced gene expression by ICP27. Available evidence demonstrates that ICP27 acts in conjunction with other viral factors to mediate the enhancement and repression of HSV gene expression. Thus, with the possible exception of the gB promoter (45), ICP27 alone is unable to either repress or enhance expression from a variety of HSV promoters tested to date. Indeed, the enhancing and repressing activities of ICP27 are apparent only in the presence of functional ICP0 or ICP4. The mapping studies described herein and elsewhere have begun to identify specific regions of the ICP27 protein involved in its enhancing and repressing activities. As noted above, the mechanism by which ICP27 represses ICP0- and ICP4-induced gene expression from promoters of the four major kinetic classes appears to be similar. With this in mind, it is of interest to note that ICP27 does not appear to affect all promoters of a given kinetic class in the same way. Indeed, in the transient expression assays of Sekulovich et al., in which a variety of promoters were tested, the expression of one early promoter (tk) was repressed by ICP27, whereas that of another (alkaline exonuclease) was stimulated (51). Likewise, the expression of one delayed early (γ1) promoter was repressed, whereas that of another was stimulated in the presence of ICP27 (52 and this study). Similarly, in infected cells, phenotypic studies of ICP27 deletion mutants have revealed that ICP27 is required to repress some early genes (e.g., ICP6 and ICP8) and activate others (e.g., ICP41) (this study). Together, these findings demonstrate that ICP27 exhibits neither specificity nor selectivity vis-a-vis the kinetic classes of viral promoters in performing its two distinct regulatory functions. Thus ICP27 alone does not play the definitive role in establishing the different kinetic classes of HSV-1 genes; rather, it is likely that other viral and cellular proteins as well as the *cis*-acting elements in viral promoters act together to determine kinetic class.

One viral protein that likely plays a pivotal role together with ICP27 in determining the kinetics of viral gene expression is ICP4. ICP4 is required to induce the expression of all early and late viral genes (9, 10, 12, 13, 43). In HSV-1 infected cells, ICP4 is synthesized as a series of phosphorylated species which vary slightly in electrophoretic mobility (13, 41, 57). Recent studies have indicated that these phosphorylated forms differ in their ability to bind to promoter sequences of HSV genes of various kinetic classes, suggesting that they play critical yet differing roles in the control of HSV gene expression (36). Cells infected with ICP27 ts (45) and deletion mutants (32; this study) produce ICP4 species with slower electrophoretic mobilities than those produced in cells infected with wild-type virus. ICP27, therefore, appears to be involved, either directly or indirectly, in the phosphorylation of ICP4. To date, however, ICP27 has not been shown to exhibit kinase activity. In studies of the vd3 mutant, a correlation was shown to exist between the absence of enhancing activity and the absence of the faster-migrating form(s) of ICP4. vd3 exhibited significant repressing activity, however, suggesting that repression does not require these faster-migrating forms. Although consistent with the findings of Michael et al. (36), this hypothesis (that ICP27 enhancing activity requires the faster-migrating form of ICP4) is in contrast to the conclusion of Hardwicke et al. (22) who stated that "differential modification of ICP4 is unlikely to account for the difference between *trans*-repression and *trans*-activation seen with ICP27." In the latter study, however, no actual test of this hypothesis was presented.

With regard to the postulated roles of different forms of ICP4 in the regulation of gene expression, it should be noted that different mutant forms of ICP4 have been shown to activate the expression of early and late genes differentially (8, 13). For example, members of the early class of ICP4 ts mutants (e.g., tsB32) underproduce both early and late proteins, while members of the late class of ICP4 ts mutants (e.g., tsB48 and ts303) induce normal levels of early proteins but reduced levels of late proteins (8). We have shown that ICP27 is absolutely required for the expression of late genes (32, 47). However, members of the late class of ICP4 mutants (i.e., tsB48 and ts303), whose genomes contain intact copies of ICP27 and ICP0, are unable to stimulate late gene expression. It is possible that the late mutant forms of ICP4 abrogate the synthesis of specific phosphorylated forms of ICP4 that are required to interact with ICP27 or ICP0 to enhance late gene expression.

Based on the evidence from this and other studies, we support a model in which ICP27 performs its regulatory activities differentially over time and in which these effects are mediated indirectly via interactions with and modifications of ICP4 and perhaps of other viral and cellular proteins.

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