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The Regions Important for the Activator and Repressor Functions of Herpes Simplex Virus Type 1 α Protein ICP27 Map to the C-Terminal Half of the Molecule

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The herpes simplex virus type 1 (HSV-1) α or immediate-early proteins ICP4 (IE175), ICP0 (IE110), and ICP27 (IE63) are *trans*-acting proteins which affect HSV-1 gene expression. We previously showed that ICP27 in combination with ICP4 and ICP0 could act as a repressor or an activator in transfection assays, depending on the target gene (R. E. Sekulovich, K. Leary, and R. M. Sandri-Goldin, J. Virol. 62:4510–4522, 1988). To investigate the regions of the ICP27 protein which specify these functions, we constructed a series of in-frame insertion and deletion mutants in the ICP27 gene. These mutants were analyzed in transient expression assays for the ability to repress or to activate two different target genes. The target plasmids used consisted of the promoter regions from the HSV-1 β or early gene which encodes thymidine kinase and from the β-γ or leaky late gene, VP5, which encodes the major capsid protein, each fused to the chloramphenicol acetyltransferase gene. Our previous studies showed that induction of pTK-CAT expression by ICP4 and ICP0 was repressed by ICP27, whereas the stimulation of pVP5-CAT expression seen with ICP4 and ICP0 was significantly increased when ICP27 was also added. In this study, a series of transfection assays was performed with each of the ICP27 mutant plasmids in combination with plasmids containing the ICP4 and ICP0 genes with each target. The results of these experiments showed that mutants containing insertions or deletions in the region from amino acids 262 to 406 in the carboxy-terminal half of the protein were unable to stimulate expression of pVP5-CAT but were able to repress induction of pTK-CAT activity by ICP4 and ICP0. Mutants in the carboxy-terminal 78 amino acids lost both activities; that is, these mutants did not show repression of pTK-CAT activity or stimulation of pVP5-CAT activity, whereas mutants in the hydrophilic amino-terminal half of ICP27 were able to perform both functions. These results show that the carboxy-terminal half of ICP27 is important for the activation and repression functions. Furthermore, the carboxy-terminal 62 amino acids are required for the repressor activity, because mutants with this region intact were able to repress. Analysis of the DNA sequence showed that there are a number of cysteine and histidine residues encoded by this region which have some similarity to zinc finger metal-binding regions found in other eucaryotic regulatory proteins. These results suggest that the structural integrity of this region is important for the function of ICP27.

During herpes simplex virus type 1 (HSV-1) infection, there at least four classes of genes which are expressed in a temporal fashion (for a review, see reference 85). The α (immediate-early) genes are expressed first, and functional α products are required for expression of later classes of genes (36, 37). The β (early) genes, which are expressed next, primarily encode products required for DNA synthesis. Transcription of β-γ (leaky-late, γ_1) genes can be detected before the onset of DNA replication; however, maximum levels of β-γ products are achieved only after DNA synthesis begins (8, 33). γ (true late, γ_2) gene products are undetectable or barely detectable in the absence of DNA replication (35, 39, 49, 76, 77, 85). Control of gene expression during HSV-1 infection appears to occur primarily at the level of transcription (40, 77, 85).

HSV-1 encodes four α or immediate proteins which have been shown to affect the expression of HSV-1 genes (36, 37). These proteins are termed ICP4 (IE175), ICP0 (IE110), ICP27 (IE63), and ICP22 (IE68). The fifth α protein, ICP47 (IE12), does not appear to affect HSV-1 gene expression (48). In this study, we extended our previous analysis of the regulatory properties of ICP27 in combination with ICP4 and ICP0, and therefore we will describe the known functions of these three α proteins in vivo and in transient expression assays.

ICP4 has been shown through analysis of temperature-sensitive (*ts*) and deletion mutants to be essential for β, β-γ, and γ gene expression in HSV-1-infected cells (10, 13, 62, 63, 86, 87). ICP4 has also been shown to autoregulate its own expression and that of other α genes (11, 13, 23, 24, 55, 58, 59, 87). The role of ICP0 during infection is not completely clear. ICP0 does not appear to be absolutely required for viral gene expression or replication (69, 73, 78); however, while ICP0 deletion mutants can be propagated on noncomplementing cell lines, these mutants grow very poorly, especially at low multiplicity, compared with the wild type (69, 78). This suggests that ICP0 confers a strong growth advantage on HSV-1. Functional analysis of ICP27 *ts* and deletion mutants demonstrated that this protein plays an essential role in virus replication (50, 68). Late gene expression was severely reduced in cells infected with ICP27 *ts* or deletion mutants, whereas early proteins were overex-

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pressed, suggesting an essential role for ICP27 in the modulation of early and late gene expression (50, 68).

In transfection assays, ICP4 has been shown to *trans*-activate HSV-1 early and late genes (11, 14, 22, 49, 57, 64, 75) but not heterologous genes (58, 59, 74). These findings, showing specificity for ICP4 action, are consistent with binding studies which have demonstrated that ICP4 binds to specific sequences in some HSV-1 promoters (2, 18, 19, 45, 46, 53, 61). Furthermore, recent evidence from Tedder and Pizer (81) suggests that ICP4 binding facilitates *trans*-activation. These investigators showed that addition of multiple tandem repeats of a consensus binding site to the early promoter from the glycoprotein D gene resulted in increased transcription in vitro in the presence of ICP4 and that ICP4 bound to the multiple binding sites (81). ICP4 also has a negative regulatory function and has been shown to repress expression from its own promoter by binding to specific regions near its cap site and in the 5′ leader (11, 24, 55, 59, 67). ICP0 is a strong promiscuous *trans*-activator in that both HSV-1 and heterologous promoters were induced in transient assays (14, 16, 23, 49, 57, 64, 74, 75). ICP0 and ICP4 can act synergistically to stimulate expression of HSV-1 early and late promoters, since greater levels of induction have been seen when both are present than with either effector alone (14, 23, 49, 57, 58, 64, 74, 75).

In contrast to the activities of ICP4 and ICP0 in transfection assays, ICP27 appears to have little or no effect on most HSV-1 or heterologous promoters (10, 14, 74). One exception is the β-γ promoter from the glycoprotein B gene, which was induced by ICP27 in studies by Rice and Knipe (65). However, while ICP27 appears to have little activity on its own, we have shown that ICP27 has two different effects in combination with ICP4 and ICP0, depending on the target gene (74). A strong *trans*-repression of the stimulation normally seen with ICP4 and ICP0 was observed in transfections with HSV-1 early target genes when all three effectors were added, whereas increased *trans*-activation was seen with a late target gene. This increased activation of a late gene by ICP27 in the presence of ICP4 and ICP0 has also been shown by Everett (15). Rice and Knipe also found that the glycoprotein B gene promoter was induced to higher levels if both ICP4 and ICP27 were present (65), and more recently, Su and Knipe (79) reported that ICP27 can inhibit the stimulation of some β target genes by ICP4 and ICP0, in accord with our earlier report (74). These results suggest that ICP27 can facilitate both down regulation and induction of HSV-1 gene expression. This is consistent with its role in vivo as demonstrated in studies with ICP27 *ts* and deletion mutants in which early gene products were overexpressed and late gene products were undetectable or greatly reduced (50, 65, 68).

ICP27 is a polypeptide of 63 kilodaltons which is phosphorylated and localizes to the nuclei of infected cells (1, 43, 89). To determine whether the repressor and activator functions of ICP27 reside in different domains of the molecule, we constructed a series of in-frame insertion and deletion mutants. The activity of each mutant was tested in transfection assays in the presence of ICP4 and ICP0 on an HSV-1 early target gene and on a late target gene. The repressor activity of ICP27 mapped to the carboxy-terminal 78 amino acids. This region contains a number of cysteine and histidine residues which bear similarity to a zinc finger metal-binding domain (3, 4). The activation function mapped to the carboxy-terminal half of the polypeptide encompassing a region of about 250 amino acids. The hydrophilic amino-terminal half of ICP27 did not appear to be essential for either activity of the protein.

MATERIALS AND METHODS

Bacteria and plasmids. *Escherichia coli* K-12 strain 1100 derivative DH-1 (29) was used as the host for propagation of all chimeric plasmids. *E. coli* C600 was the host strain used in expression studies with the pATH fusion vectors (7, 80). The effector plasmids pSG28K/B, which contains the ICP4 gene; pRS1, which contains the ICP0 gene; and pSG130B/S, which contains the ICP27 gene, have been described previously (74). The construction of the ICP27 insertion and deletion mutants is described in the text. All of the mutants were sequenced around the site of the inserted linker. Plasmid pIEP, which contains immediate-early protein IEP from pseudorabies virus (PRV), was provided by L. Feldman (7). The target plasmids pTK-CAT and pgD-CAT were described previously (74). pVP5-CAT was provided by E. K. Wagner (5). The *trp* expression vectors pATH1, pATH2, and pATH3 were given to us by A. Tzagoloff (80). Plasmid pICP4-pATH1 was constructed by cloning a 1,737-base-pair (bp) *Bam*HI-*Nco*I fragment from the ICP4 gene into the *Bam*HI and *Hin*dIII sites of pATH1. The *Hin*dIII and *Nco*I sites were filled by Klenow before ligation. This results in in-frame expression of a portion of the ICP4 protein from amino acids 564 to 1144 (52) fused to the first 336 residues of the *E. coli* Trp protein (7, 80). Plasmid pICP27-pATH2 consists of a 678-bp *Hin*fI-*Sal*I fragment from the amino-terminal half of the ICP27 gene inserted into the *Bam*HI and *Sal*I sites of pATH2. The *Hin*fI site was first modified by treatment with Klenow and ligation of *Bgl*II linkers. This resulted in in-frame expression of amino acids 39 to 262 of ICP27 fused to Trp. Plasmid pICP27-pATH3 was constructed by insertion of a 1,250-bp *Sal*I-*Sst*I fragment of the ICP27 gene into the *Sal*I and *Hin*dIII sites of pATH3. The *Sst*I and *Hin*dIII sites were modified with Klenow before ligation. This results in in-frame expression of amino acids 262 to 512 of the ICP27 protein fused to Trp. ICP27 deletion and insertion mutants H7, H17, S1B, and N2 were similarly cloned in pATH3. Expression of these fusion peptides is described in Results.

Production of antibodies. Synthetic oligopeptide NH_2-V-H-G-L-Y-P-Y-C-N-S-L-F-COOH (synthesized at the Protein Synthesis Facility at the University of California San Diego) corresponds to the C-terminal 12 amino acids in the ICP27 peptide predicted from the DNA sequence (51). The peptide was coupled to bovine serum albumin, and antiserum was raised in rabbits. Antibodies specific for ICP27 were selected by passing the sera through a Sepharose column to which the synthetic peptide (without bovine serum albumin) had been coupled. Antibodies to *E. coli* Trp fusion proteins were generated by expressing the ICP4-pATH1, ICP27-pATH2, and ICP27-pATH3 fusion constructs in C600 cells by induction with indoleacrylic acid (7). Whole-cell lysates were fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gels as described below. The fusion proteins were identified by the presence of a band with the predicted molecular weight in the chimeric plasmids and the disappearance of the Trp protein band seen with the pATH vectors alone. The fusion proteins were further identified by reaction of the fusion products in Western blots (immunoblots) with an anti-Trp antibody which was kindly provided by L. Feldman. The ICP4-Trp and ICP27-Trp fusion protein bands were excised from gels, and the proteins were electroeluted. Rabbits were injected by standard

protocols (71). The antibodies raised to the fusion proteins were shown to react in Western blots with the corresponding HSV-1 protein from infected cell lysates compared with reactions with an ICP4-monospecific antibody kindly provided by K. Wilcox (19) and an ICP27 monoclonal antibody kindly provided by L. Pereira (1).

Cells and transfection. Rabbit skin fibroblasts obtained from the American Type Culture Collection were grown as described previously (74). For transfection experiments in which chloramphenicol acetyltransferase (CAT) activity was assayed, transfections were performed in 35-mm-diameter six-well cluster dishes as described previously (74). The target plasmids were added at 20 μg of plasmid DNA per well, whereas the effector plasmids were added at equimolar ratios for most experiments; that is, ICP4 plasmid pSG28K/B was added at 5 μg/ml, ICP0 plasmid pRS1 was added at 2.5 μg/ml, and ICP27 plasmid pSG130B/S was added at 1.25 μg/ml of transfection buffer. In some experiments, the wild-type and mutant ICP27 plasmids were added at different concentrations, and these are described in the figure legends. The total amount of DNA was adjusted to 30 μg/ml by addition of pUC18 DNA. Cells were harvested 48 h after transfection. CAT activity was measured by a diffusion assay as described by Neumann et al. (56), except that 0.5 μCi of ^{3}H-acetyl coenzyme A was used in each reaction. CAT activity, measured by the diffusion assay, was compared with that measured by the assay described by Gorman et al. (27), and the results were found to be equivalent in a series of five different experiments. In all subsequent experiments, the diffusion assay was used because it allows rapid processing of large numbers of samples.

For analysis of expression of ICP4 by immunoprecipitation, subconfluent monolayers of rabbit skin fibroblasts in 75-cm^2 flasks were transfected with 20 μg of target plasmid DNA per ml. Effector plasmids were added at equimolar ratios. A total volume of 5 ml of transfection solution was added to each flask. Cells were shocked with glycerol 5 h after transfection as described previously (74). Transfected cultures were labeled with 100 μCi of $^{32}P_i$ per flask in 4 ml of phosphate-free medium (GIBCO Laboratories) containing 1% fetal bovine serum. Cells were labeled for 7 h, beginning at 24 h after transfection. Labeled cells were harvested as described by Holland et al. (34).

For immunofluorescence studies on the cellular localization of ICP27, COS cells (26) were transfected with ICP27 wild-type or mutant plasmids as described in Results, by using lipofectin reagent (20; Bethesda Research Laboratories, Inc.). Cells were seeded on glass cover slips in 35-mm-diameter dishes. Plasmid DNA (20 μg per well) diluted in Opti-MEM (GIBCO) was mixed with lipofectin as recommended by the supplier and then added to the cover slips dropwise. After 24 h, the lipofectin mixture was removed and fresh medium containing 10% fetal bovine serum was added. After 48 h, cells were washed in phosphate-buffered saline containing 1% calf serum and fixed as described by Everett (16), except that 3.7% instead of 2% formaldehyde was used. Cells were then preadsorbed with a 1:50 dilution of normal donkey serum (Accurate Chemical Scientific Corp.). Cells were incubated with rabbit antiserum to the ICP27-Trp fusion protein specified by ICP27-pATH2. Cells were then incubated with anti-rabbit immunoglobulin whole antibody from a donkey. Finally, the cells were treated with fluorescein-streptavidin (Amersham Corp.). The stained cells were examined by epifluorescence microscopy.

Immunoprecipitation, polyacrylamide gel electrophoresis, and Western blotting procedures. Immunoprecipitations with antibody to the ICP4-Trp fusion protein were performed as described previously (70). Polyacrylamide gel electrophoresis was performed as described by Vaughan et al. (83). Immunoblotting experiments were done by a slight modification of the method of Towbin et al. (82). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose was done by using the Bio-Rad Laboratories Trans-Blot system. Transfer took place in a buffer of 40% methanol–25 mM Tris–190 mM glycine (pH 8.5) at 30 V overnight. After transfer, the nitrocellulose was soaked in a solution of 3% glycine–0.9% NaCl–10 mM Tris hydrochloride (pH 7.5)–10% calf serum–0.1% Tween 20 to block the remaining protein-binding sites. After being washed, the nitrocellulose was reacted with the specific antisera diluted in phosphate-buffered saline containing 10% calf serum. The blot was then washed and reacted with alkaline phosphatase-conjugated goat anti-rabbit serum. A color development reagent containing 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue Tetrazolium was used to visualize the proteins. The blot was washed in distilled water and dried.

RESULTS

ICP27 does not alter the level of ICP4 in cotransfections. We previously showed that ICP27 down regulated the level of ICP0 RNA along with the level of CAT RNA in transfections with three different HSV-1 target plasmids (74). These target plasmids consisted of HSV-1 promoter sequences from early and late genes fused to the gene that encodes CAT. However, we further showed that the level of induction of an HSV-1 target plasmid was not directly proportional to the amount of input ICP0 plasmid or to the amount of ICP0 mRNA expressed (74). Therefore, the mechanism of down regulation of ICP27 could not be explained simply by down regulation in the level of ICP0. Furthermore, ICP27 acted with ICP4 and ICP0 to activate some promoters. As a first step in analyzing the activities of ICP27, we wished to investigate whether any differences in ICP4 expression occurred in the presence of ICP27 in transfections with a target plasmid which is repressed by ICP27 compared with one which is normally stimulated.

The target plasmids used included pTK-CAT, which contains the early promoter from the HSV-1 thymidine kinase gene and is repressed by ICP27, and pVP5-CAT, which contains the promoter from β-γ gene VP5, which encodes the major capsid protein and is stimulated in the presence of ICP27 (74). Each of the target plasmids was transfected with ICP4, ICP0, and ICP27 effector plasmids. Because ICP4 is a phosphoprotein (89), cells were labeled with ^{32}P for 7 h, beginning at 24 h after transfection. Cell lysates were immunoprecipitated with a monospecific antibody to ICP4 which was raised against an ICP4-Trp bacterial fusion protein (see Materials and Methods). The autoradiograph of the polyacrylamide gel is shown in Fig. 1. The amount of ICP4 detected in the transfections appeared to be equivalent for both the pTK-CAT and pVP5-CAT targets (lanes 2 and 3). CAT assays performed on samples of the lysates showed that pTK-CAT activity was repressed, as expected, when ICP27 was included in the transfections and pVP5-CAT activity was stimulated (data not shown). Therefore, although expression of the target plasmid was affected by the presence of ICP27, expression of ICP4 was not (Fig. 1).

Su and Knipe (79) have reported a mobility shift in denaturing polyacrylamide gels for ICP4 from cells transfected with plasmids that encode ICP4 and ICP27 compared with cells transfected with ICP4 alone. This suggests that

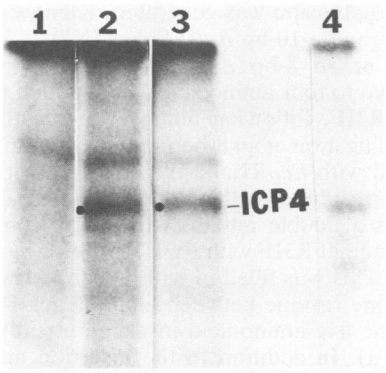

FIG. 1. Expression of ICP4 in the presence of ICP27 with two different target plasmids. To determine whether ICP4 expression was different with a target plasmid that was repressed in the presence of ICP27 from that with one which was activated, transfections were performed with pTK-CAT or pVP5-CAT as the target, each at a concentration of 20 μg/ml. Lanes: 1, pTK-CAT (20 μg/ml) transfected with the effector plasmids pRS1 (ICP0; 2.5 μg/ml) and pSG130B/S (ICP27; 1.5 μg/ml) as a negative control for expression of ICP4; 2, pTK-CAT transfected with pRS1, pSG130B/S, and pSG28K/B (ICP4; 5 μg/ml); 3, pVP5-CAT transfected with pRS1, pSG130B/S, and pSg28K/B (ICP4). Cells were labeled with ^{32}P for 7 h, beginning at 24 h after transfection. Cells were harvested and immunoprecipitated with antibody to ICP4 which was raised against an ICP4-Trp fusion protein as described in Materials and Methods. Immunoprecipitates were fractionated on an SDS-polyacrylamide gel as previously described (83). The autoradiograph also shows a lane (lane 4) on which a ^{32}P-labeled infected-cell lysate was run. HSV-1 infection was performed under conditions which maximize the expression of α proteins (90).

ICP27 can modify ICP4 in some way. We detected no qualitative alterations in ICP4 in this experiment using two different target plasmids (Fig. 1, lanes 2 and 3). This indicates that while ICP27 may modify ICP4 in some way (65, 79), the mobility of ICP4 in the presence of ICP27 was not changed when a target which was repressed was used rather than one which was activated. Therefore, differential modification of ICP4 is unlikely to account for the difference between *trans*-repression and *trans*-activation seen with ICP27.

ICP27 can interact with another herpesvirus *trans*-activator. The finding that ICP27 had little activity on target genes on its own but displayed repressor or activator functions in the presence of ICP4 and ICP0 (74) suggests that ICP27 acts cooperatively in some way with these α proteins. ICP4 and ICP0 can act synergistically in transfections, and Everett (16, 17) has identified regions of the ICP0 protein which appear to be involved in the interaction of ICP0 with ICP4. To determine whether ICP27 interacts specifically with ICP4 and ICP0, we performed a series of transfection experiments in which a plasmid that encodes the immediate-early protein from PRV (pIEP) was substituted for the ICP4 plasmid. The target plasmids used were pgD-CAT, which we previously showed was down regulated in the presence of ICP4, ICP0, and ICP27 (74), and pVP5-CAT. The effector plasmids were added in equimolar ratios either singly or in combination. Figure 2 shows the results of these experiments. In these and all of the following experiments, the results are presented as fold induction over the activity seen with the target plasmid in the presence of pUC18 with no effector added. This uninduced value was always derived from at least four separate transfections in each set of assays and was arbitrarily set at 1.0. The fold induction for transfections with effector plasmids was calculated relative to this value. To control for variations in transfection efficiency, we performed the experiments a number of times. The means are plotted, and standard errors of the means are shown as error bars.

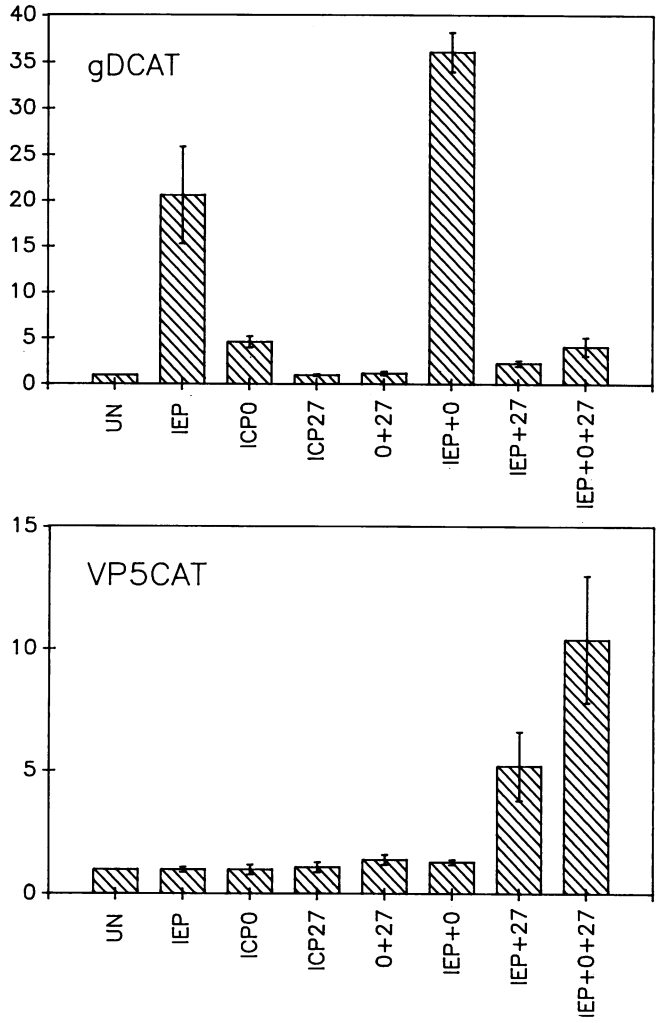

FIG. 2. Effect of ICP27 on expression of HSV-1 target plasmids in the presence of PRV IEP. Two different target plasmids, pgD-CAT and pVP5-CAT (20 μg/ml), were cotransfected with pIEP, which contains the immediate-early protein from PRV (5 μg/ml), pRS1 (ICP0; 2.5 μg/ml), or pSG130B/S (ICP27; 1.5 μg/ml). The effector plasmids were added separately or in combination, as indicated in each panel. The data are presented as fold induction relative to the value obtained in the uninduced samples (UN). This uninduced value was obtained from four separate transfections in each experiment with the target plasmids and pUC18 DNA (10 μg/ml) in the absence of effectors. The uninduced value was set equal to 1.0. Each transfection was performed at least six times to control for variability. Error bars are shown.

IEP induced pgD-CAT and acted synergistically with ICP0, demonstrating that ICP0 can interact functionally with PRV IEP (Fig. 2). ICP27 down regulated the induction of pgD-CAT by IEP in the presence or absence of ICP0. Therefore, IEP behaved similarly to HSV-1 ICP4 and ICP0 in its effect on pgD-CAT (Fig. 2). IEP also behaved similarly in its effect on pVP5-CAT in that little induction of pVP5-CAT was seen with IEP alone or with IEP and ICP0; however, induction of pVP5-CAT was seen when ICP27 was

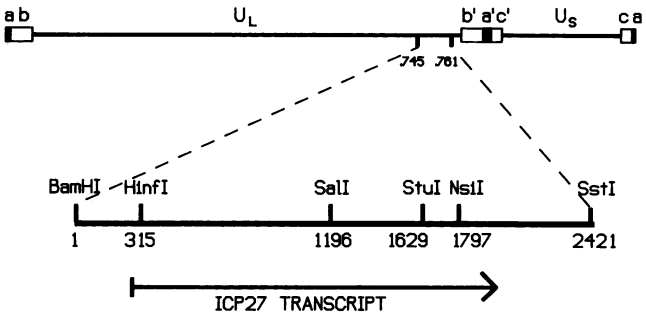

FIG. 3. Schematic representation of the HSV-1 genome showing the relative position of the ICP27 gene. The ICP27 gene maps to the unique long (U_L) segment of the HSV-1 genome between coordinates 0.745 and 0.761. The ICP27 gene was cloned as a *Bam*HI-*Sst*I fragment of 2421 bp in the vector pUC18 and was designated pSG130B/S (74). The insertion and deletion mutants were constructed from this plasmid. ICP27 has been sequenced (51), and in this representation, the *Bam*HI site is designated as nucleotide 1 and the *Sst*I site is nucleotide 2421. Other restriction sites are given for orientation. The position of the 1.8-kilobase unspliced transcript is shown (88). The transcript initiates at nucleotide 275. U_S, Unique short segment. a, b, c, a', b', and c' indicate the inverted repeat region.

added to the transfections (Fig. 2). These results demonstrate that ICP27 can act cooperatively with another *trans*-activator, IEP, in addition to ICP4 and ICP27.

Identification of the repressor and activator regions of ICP27. ICP4 and E1A from adenovirus have both been shown to function as activators or as repressors (6, 11, 24, 25, 31, 32, 38, 42, 58, 59, 84, 87). Mutational analyses of the ICP4 and E1A genes have shown that activator and repressor activities reside in specific regions of these proteins (12, 54, 60). To determine whether the activator and repressor functions of ICP27 are encoded in the same region or in different domains of the molecule, we constructed a series of in-frame insertion mutants in the ICP27 plasmid. We also isolated four in-frame deletion mutants. The ICP27 mutants were tested for activities in transfection experiments in the presence of ICP4 and ICP0 on two different target genes, pTK-CAT and pVP5-CAT.

The ICP27 gene is encoded within the long unique region of the HSV-1 genome from coordinates 0.745 to 0.761 (Fig. 3). The ICP27 gene has been sequenced by McGeoch et al. (51). In this representation, the *Bam*HI site has been designated as nucleotide 1 and the *Sst*I site is nucleotide 2421. The TATA box homology occurs at nucleotide 247, and the transcript initiates at nucleotide 275. The coding sequence, which begins at the ATG at nucleotide 413 and terminates at a TAG at position 1949, encodes a protein of 512 amino acids (51). In ICP27 plasmid pSG130B/S, the *Bam*HI-*Sst*I fragment was cloned into pUC18 as described previously (74). Mutants were constructed by digestion with restriction enzymes which cut the ICP27 plasmid at a single site, or if multiple sites were present, partial digestions were performed to generate linear molecules. Synthetic restriction enzyme linkers were introduced into each site after appropriate modification so that each insertion would be in frame. The insertion sites and the linkers inserted are listed in Table 1. When the restriction enzyme generated blunt ends, a 12-bp *Eco*RI or *Bgl*II linker was inserted. When a four-base 5' extension was produced (Table 1, S13), the ends were filled in with Klenow and an 8-bp *Eco*RI linker was ligated to the molecules. Similarly, if a four (N2)-, three (D2)-, or two (S23)-base 3' extension was generated, Klenow was used to trim the ends and a 10-bp *Bgl*II linker (N2), a 12-bp *Eco*RI linker (D2), or an 8-bp *Eco*RI linker (S23) was added. Therefore, two to four amino acids were added to each site. For mutant R3IF, following filling in of the three-base 5' overhang and ligation of an 8-bp *Eco*RI linker, the molecules were digested with *Eco*RI and then filled in with Klenow a second time, resulting in the addition of five amino acids. Mutant R1 is a double mutant which was constructed by digestion of mutant R3IF with *Rsr*II, resulting in a three-base 5' overhang. This was filled in with Klenow, resulting in an insertion of one residue between amino acids 58 and 59, in addition to the five-amino-acid insertion already present in R3IF (Table 1). In addition to the insertion mutants, four in-frame deletion mutants were isolated by this procedure (S5, H7, H17, and F35). These mutants were the products of partial digestions in which the restriction enzyme cut at more than one site. The extents of the deletions are given in Table 1. All mutants were sequenced around the site of the linker insertion to be sure that the coding sequence remained in frame. In three instances (N9M, S13M, and B7M), multiple insertions of the linker sequence were found. These mutants were included in the analysis and are indicated in Table 1. The additional linkers in S13M and B7M were removed by restriction enzyme digestion and religation of the plasmid DNA. The mutants with single linker inserts (S13 and B7) were similarly sequenced and tested in transfection experiments.

Transfections were performed with target plasmids pTK-CAT and pVP5-CAT. ICP4 and ICP0 plasmids were added to the transfections along with the wild-type or mutant ICP27 plasmids. We showed previously that ICP27 exerted its effect as a repressor or activator in the presence of ICP4 and ICP0 over a wide range of input plasmid concentrations (74). In the experiments presented here, the ICP4 and ICP0 plasmids were added at equimolar concentrations. The ICP27 wild-type and mutant plasmids were added to the transfections at three different concentrations over a fivefold range (see the legend to Fig. 4). Very little difference was seen in the magnitude of the effect of the ICP27 mutants at these different plasmid concentrations, and therefore the results of the separate transfections are combined in the data presented in Fig. 4 and Table 1.

All of the mutants except B7, N2, S2, and S18 repressed the stimulation of pTK-CAT seen with ICP4 and ICP0 (Fig. 4; Table 1). These four mutants, which encompass the region from amino acids 434 to 505 in the carboxy terminus of the protein, failed to repress the stimulation of pTK-CAT by ICP4 and ICP0. Therefore, it is likely that the domain of ICP27 which is involved in repression resides in this carboxy-terminal region. This is further supported by the finding that deletion mutants H7 and H17 were able to repress the stimulation of pTK-CAT (Fig. 4). These mutants contain deletions of 66 and 156 amino acids, respectively, in the carboxy-terminal half of the protein, but both encode the carboxy-terminal 62 amino acids (Table 1).

The region involved in activation of pVP5-CAT by ICP27 in combination with ICP4 and ICP0 appears to encompass the carboxy-terminal half of the protein. Mutants with insertions before residue 262 were able to activate pVP5-CAT, whereas all of the mutants from residues 262 to 505 failed to activate the target plasmid (Fig. 4; Table 1). Mutants in the region from amino acids 262 to 406 repressed pTK-CAT, and therefore these mutants are defective only in the ability to activate the late target gene in synergy with ICP4 and ICP0. Mutants in the carboxy terminus from amino acids 434 to 505

TABLE 1. Summary of positions and activities of ICP27 insertion and deletion mutations

Mutant	Nucleotide insertion site, enzyme[a]	Amino acid residues[b]	Inserted amino acid sequence(s)[c]	Fold induction (no. of expts)[d]	
				pTK-CAT	pVP5-CAT
No ICP27				31.8 ± 4.8 (12)	2.2 ± 0.4 (8)
Wild-type ICP27				2.4 ± 1.5 (34)	18.5 ± 3.3 (30)
N9M	490, *Nae*I	27/28	RNSGRNSG	1.5 ± 0.5 (8)	12.5 ± 2.2 (6)
R1	584, 619, *Rsr*II	58/59; 70/71	R; VGINS	1.2 ± 0.2 (6)	10.4 ± 2.1 (6)
R3IF	619, *Rsr*II	70/71	VGINS	1.2 ± 0.6 (8)	13.9 ± 2.5 (15)
D2	721, *Dra*III	102/104	−S; +PEFR	3.5 ± 1.4 (9)	7.4 ± 1.1 (8)
S5	871–946, *Sma*I	153–178	−25 aa; +PEFR	4.2 ± 0.7 (6)	7.4 ± 1.2 (6)
N6	900, *Nae*I	163/164	R→P; GIPG	5.2 ± 1.1 (11)	14.4 ± 2.6 (6)
F20	996, *Fnu*DII	195/196	EDLP	0.8 ± 0.1 (8)	5.7 ± 1.0 (13)
F21	1104, *Fnu*DII	231/232	EDLP	3.4 ± 1.2 (6)	18.5 ± 2.8 (6)
S13M	1196, *Sal*I	262/263	EEFRNSGIPEPL	2.4 ± 0.5 (11)	1.5 ± 0.2 (13)
S13	1196, *Sal*I	262/263	EEFL	3.4 ± 1.9 (6)	1.6 ± 0.3 (6)
H17	1274–1745, *Fnu*DII	288–444	−156 aa; +AGIP	4.7 ± 1.1 (9)	1.2 ± 0.2 (11)
N21	1391, *Nae*I	327/328	PEFR	3.2 ± 1.3 (13)	1.1 ± 0.1 (6)
H7	1562–1760, *Fnu*DII	383–450	−66 aa;+AAGIP	3.3 ± 0.6 (12)	1.2 ± 0.1 (11)
S23	1563, *Sst*II	383/384	A→G; IP	1.5 ± 0.4 (6)	1.2 ± 0.2 (8)
S1B	1629, *Stu*I	406/407	RNSG	1.5 ± 0.5 (6)	1.6 ± 0.5 (9)
B7M	1712, *Bal*I	434/435	GIPAGIPAGIPA	23.7 ± 6.2 (8)	1.3 ± 0.1 (8)
B7	1712, *Bal*I	434/435	GIPA	32.8 ± 4.2 (6)	1.5 ± 0.1 (6)
F35	1745–1760, *Fnu*DII	445–449	A→A; GIP	ND	ND
N2	1797, *Nsi*I	459/460	MH→RR; SS	25.5 ± 4.3 (9)	1.3 ± 0.5 (8)
S2	1807, *Sma*I	465/466	PEFR	14.3 ± 2.9 (6)	1.2 ± 0.3 (6)
S18	1925, *Ssp*I	504/505	Y→S; FIPR	14.6 ± 2.7 (9)	1.2 ± 0.1 (12)

[a] The coordinate given is the position of the first base in the recognition sequence of the restriction enzyme insertion site in the DNA sequence of the ICP27 gene (51); *Bam*HI is designated nucleotide 1, and *Sst*I is designated nucleotide 2421. The inserted oligonucleotides were as follows: CCGGAATTCCGG for mutants N9M, D2, S5, N6, H17, N21, H7, S1B, B7M, B7, F35, S2, and S18; GGAAGATCTTCC for mutants F20 and F21; GGAATTCC for mutants R31F, S13M, S13, and S23; and GAAGATCTTC for mutant N2.

[b] The insertions lie between the two residues shown. For deletion mutants S5, H17, H7 and F35, the residues deleted are given. For mutant R1, two insertions were made: R between residues 58 and 59 and VGINS between residues 70 and 71.

[c] The inserted amino acids (aa) are given. When the insertion substituted a residue, as in S23, N2, and S18, this is indicated as A→G, etc. For deletion mutants S5, H17, H7, and F35, the number of deleted amino acids is indicated as well as the inserted amino acids.

[d] Mean values of induction from the stated number of experiments are presented as fold induction relative to the value obtained with the target plasmid in the presence of pUC18. This level was set as 1.0. The target and effector plasmids were added at the concentrations stated in the legend to Fig. 4. In all cases, the mutants were added to transfections in the presence of the ICP4 and ICP0 plasmids. ND, Not done. Mutant F35 has a *ts* phenotype. Results of transfections with F35 at 34 and 39°C are presented in Fig. 6.

are also defective in the repressor function. This suggests that the region from residues 262 to 406 is important for activation, whereas the region from 434 to 505 is important for both repression and activation. Mutants in the amino-terminal half of the protein from amino acids 27 to 232 retained the ability to activate and to repress; thus, this region does not appear to be essential for either function.

The mutants in the region from 434 to 505 were defective in both activities of ICP27, and therefore it was possible that these mutants were defective in nuclear localization. If that were the case, the mutant peptides would not be transported to the nucleus and therefore neither function would be carried out by the cytoplasmic peptides. Analysis of the predicted amino acid sequence of the carboxy-terminal region of ICP27 (51) revealed no sequences which were similar to the nuclear localization signals defined in other nuclear proteins, such as simian virus 40 and polyomavirus T antigens (41, 66) or ICP4 (12) and ICP0 (17). To determine the cellular location of the mutant peptides, cells were transfected with wild-type ICP27 plasmid or with mutant B7 or S18 and then treated with an antibody specific for ICP27 and observed by immunofluorescence staining. Mutant B7 contains an insertion of four amino acids between residues 434 and 435, and S18 contains a change at residue 504 of a tyrosine to a serine and an insertion of four amino acids (Table 1). Both mutants were defective in activation and repression (Fig. 4).

To increase the level of expression of ICP27 in these transfections, the wild-type and mutant plasmids were modified by addition of the simian virus 40 origin of replication and transfections were performed in COS cells which express simian virus 40 T antigen (26). This resulted in amplification of the plasmid copy number in each transfected cell. A 520-bp *Acc*I-*Hin*dIII fragment from pSV2-CAT (27) containing the SV40 early promoter and origin of replication was cloned into the *Hin*dIII site of the wild-type ICP27 plasmid and the mutant plasmids B7 and S18. The *Hin*dIII site is in the polylinker of vector pUC18 and occurs upstream of the ICP27 promoter. COS cells were transfected by using lipofectin as described in Materials and Methods to increase the percentage of transfected cells (20). Cells were fixed after 48 h and incubated with an antibody raised against the ICP27-Trp fusion protein produced by ICP27-pATH2 which encodes residues 39 to 262 (Materials and Methods). Approximately 5 to 10% of the cells expressed ICP27, and the staining seen in the expressing cells was predominantly nuclear for wild-type ICP27 (Fig. 5B) and for B7 (Fig. 5C) and S18 (Fig. 5D). A low background level of staining was observed in cells transfected with pUC18 alone (Fig. 5A), but this staining was not concentrated in the nucleus. The nuclear staining observed for ICP27 for both the wild-type and mutant proteins was evenly distributed over the nucleus but was not seen in the nucleoli, similar to what has been observed for ICP27 in infected cells (43). These results demonstrate that ICP27 expressed by B7 and S18 was efficiently transported to the nucleus. Furthermore, we have recently constructed a mutant in the amino-terminal half of the protein in an arginine-rich region which is similar to the

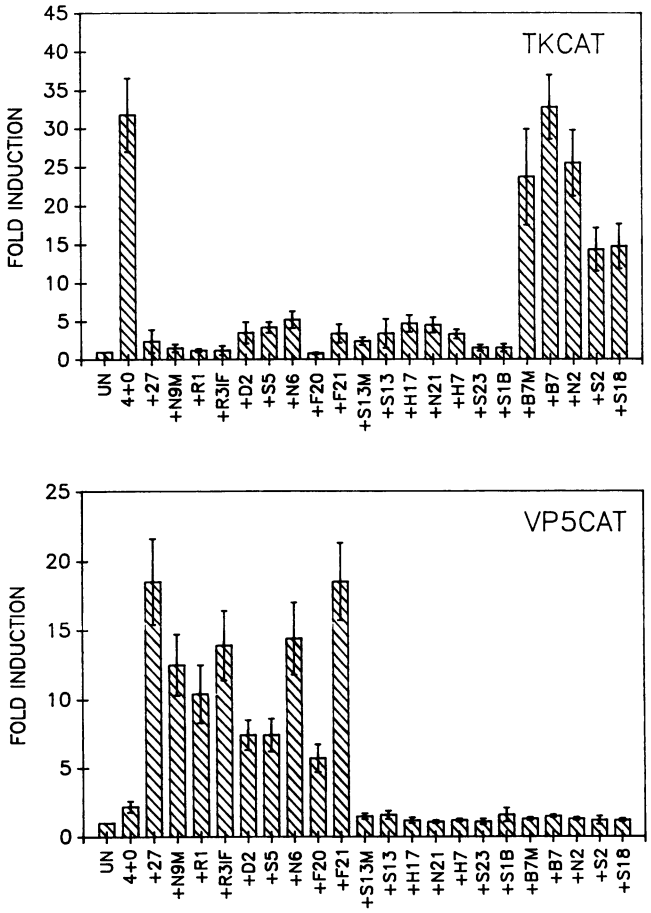

FIG. 4. Effects of insertion and deletion mutations in the ICP27 gene on the repressor and activator functions of the protein. Target plasmids pTK-CAT and pVP5-CAT (20 μg/ml) were cotransfected with each of the ICP27 insertion and deletion mutant plasmids in the presence of the ICP4 plasmid (pSG28K/B; 5 μg/ml) and the ICP0 plasmid (pRS1; 2.5 μg/ml). Transfections were also performed with the targets in the absence of effectors (UN), with ICP4 and ICP0 (4+0), and with ICP4, ICP0, and the wild-type ICP27 plasmid pSG130B/S (indicated as +27). Results are presented as fold induction over the value obtained for the uninduced samples, which was set equal to 1.0. Mean induction values are shown, and each transfection was performed at least six times. The wild-type and mutant ICP27 plasmids were added to the transfections over a range of concentrations from 0.25 to 1.25 μg/ml. Little variation was seen in the magnitude of induction at these different concentrations within each set of experiments, and therefore all of the values shown are averages. The standard errors of the means are shown as bars.

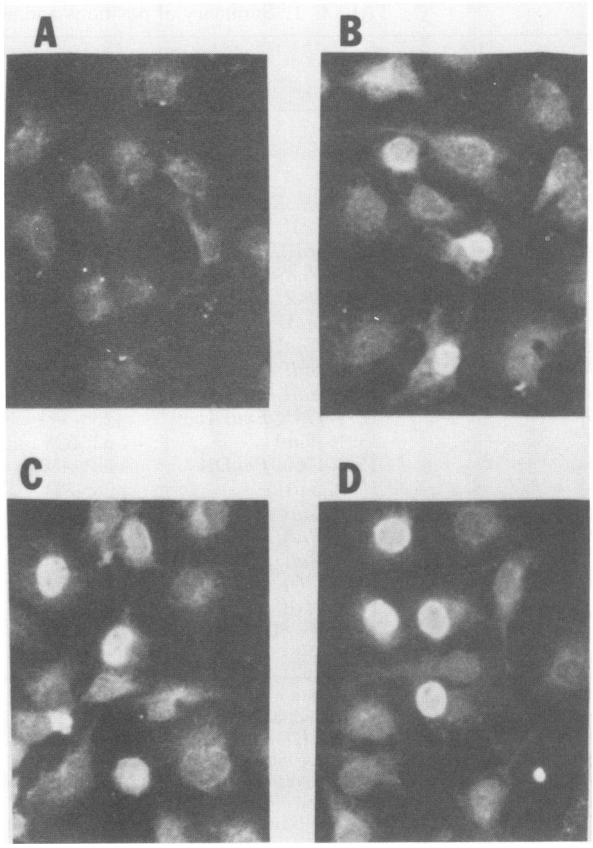

FIG. 5. Immunofluorescence analysis of the cellular location of wild-type and mutant ICP27. COS cells were transfected with the wild-type ICP27 plasmid pSG130B/S (B) or with mutant plasmid B7 (C) or S18 (D) at a concentration of 20 μg/ml. The simian virus 40 origin of DNA replication had been inserted into each plasmid so that the plasmid copy number would be amplified in the transfected COS cells to increase expression of ICP27 (26). Panel A shows COS cells transfected with pUC18 (20 μg/ml) as a control for background staining. Cells were fixed 48 h after transfection and treated with an antibody raised against a bacterially expressed ICP27-Trp fusion protein.

proposed nuclear localization region in ICP0 (17). This mutant is defective in both activation and repression, and the mutant protein was not found in the nuclear fraction of transfected cells (M. A. Hardwicke, P. J. Vaughan, and R. M. Sandri-Goldin, manuscript in preparation). This result further indicates that the nuclear localization signal does not reside in the carboxy terminus of the protein.

Identification of a *ts* repressor and activator mutant. In the insertion mutants described above, two to four amino acids were added at each site (Table 1). Therefore, it was possible that some of the mutants would display a *ts* phenotype. The mutants were tested in transfection assays as described earlier, but the transfections were incubated at 34 and 39°C. In addition, the *ts* ICP27 locus from the HSV-1 mutant *ts*LG4 (72) was cloned as a *Bam*HI-*Sst*I fragment in pUC18 and included in these experiments. Expression of late gene products is severely reduced in infections with *ts*LG4 at the nonpermissive temperature (65, 68).

Only one of the mutants isolated in this study displayed a *ts* phenotype in these assays, mutant F35. While this mutant was able to repress the stimulation of pTK-CAT by ICP4 and ICP0 at 34°C as efficiently as the wild-type ICP27 plasmid and LG4, it failed to repress at 39°C (Fig. 6). In F35, 15 bp were deleted between nucleotides 1745 and 1760 and a 12-bp *Eco*RI linker was added, resulting in an insertion of four amino acids between amino acids 445 and 449. This site lies in the region from residues 434 to 505 which was shown above to be involved in the repressor function of ICP27 (Table 1; Fig. 4). This region was also found to be important for activation. Therefore, we tested the ability of F35 and LG4 to activate pVP5-CAT in synergy with ICP4 and ICP0 at 34 and 39°C. Both mutants activated pVP5-CAT at 34°C, although less efficiently than did the wild-type ICP27 plasmid; however, neither mutant activated VP5-CAT at 39°C (Fig. 6). Therefore, F35 is *ts* in both the repressor and activator functions. These data support the notion that the

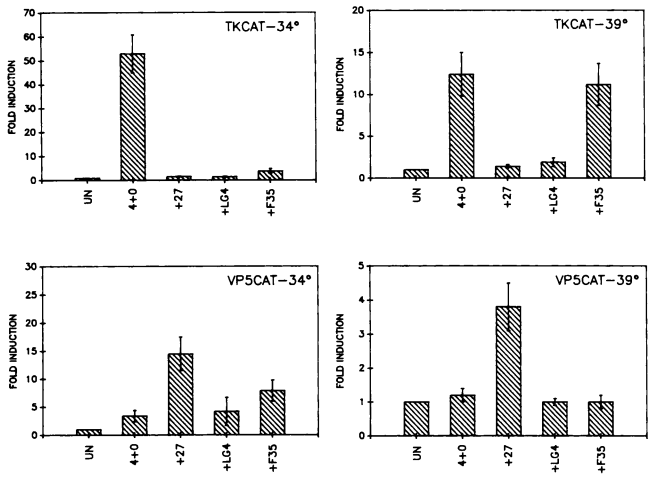

FIG. 6. Mutant F35 is *ts* for repression and activation. The ICP27 insertion and deletion mutants were tested for *ts* phenotypes by performing transfections with pTK-CAT and pVP5-CAT in the presence of ICP4 and ICP0 at 34 and 39°C. In addition, the locus from the ICP27 *ts* mutant *ts*LG4 was cloned as a *Bam*HI-*Sst*I fragment in pUC18 and the plasmid designated LG4 was included in the transfection experiments. Values presented as fold induction are means of induction of 9 to 11 separate transfections in each case. Error bars are shown. The uninduced value obtained with the target plasmids without effectors (UN) was set to 1.0. In all other transfections, the effector plasmids encoding ICP4 and ICP0 were present at the concentrations indicated in the legend to Fig. 4. The wild-type ICP27 plasmid and the F35 and LG4 mutants were added at a concentration of 1 μg/ml.

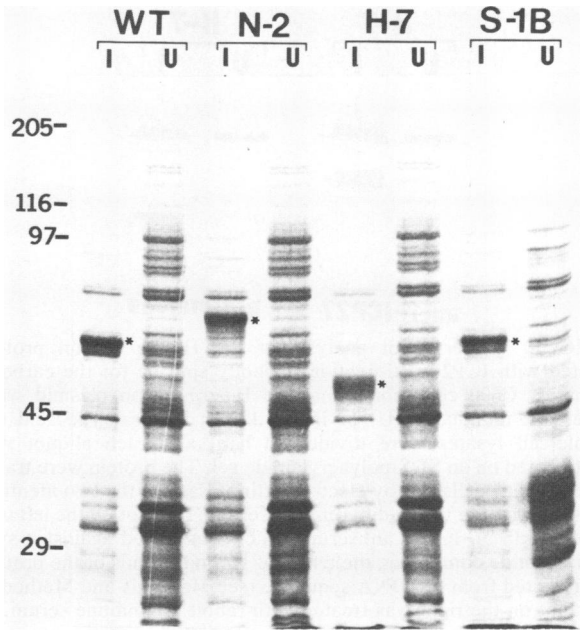

FIG. 7. Expression of ICP27 mutants as Trp fusion proteins in *E. coli*. *Sal*I-*Sst*I fragments from wild-type ICP27 plasmid pSG130B/S and from mutants N2, H7, and S1B were cloned into bacterial expression vector pATH3 (7, 80). This results in expression of the carboxy-terminal half of the ICP27 protein from amino acid 262 fused in frame to the amino-terminal 332 amino acids of the Trp protein. C600 cells containing the Trp fusion plasmids were induced with indoleacrylic acid. Whole-cell lysates were fractionated on an SDS–10% polyacrylamide gel which was stained with Coomassie blue to detect the proteins. The position of the each fusion peptide in the induced samples (I) is shown by an asterisk. Fusion peptides were not detected in uninduced samples (U). The numbers on the left indicate molecular sizes in kilodaltons.

carboxy-terminal 71 amino acids from residues 434 to 505 are important for both the repressor and activator functions of ICP27. These results also show that LG4 is defective only in the activator function. The exact site of the alteration in LG4 was not determined. Marker rescue analysis of the viral mutant *ts*LG4 has shown that the site of the lesion is within the middle of the ICP27 gene (I. L. Smith and R. M. Sandri-Goldin, unpublished data). This is consistent with the positions of the insertion and deletion mutants which affect activation but not repression. These mutants map between residues 262 and 406 (Table 1).

The carboxy-terminal 62 amino acids are required for repressor activity in ICP27. Mutants H7 and H17 were defective in the activator function of ICP27, but both mutants were able to repress stimulation of pTK-CAT by ICP4 and ICP0 (Fig. 4). These mutants have relatively large deletions in the carboxy-terminal half of the protein, deletions of 66 and 156 amino acids, respectively. Sequence analysis of these mutants showed that each retained the terminal 62 amino acids in the correct reading frame. To be certain that H7 and H17 express truncated peptides with the carboxy terminus intact, we looked at the proteins made by these mutants. For this purpose, we used an antibody which was generated against a synthetic peptide consisting of the terminal 12 amino acids of ICP27. Detection of the H7 and H17 proteins by this antibody would show definitively whether the carboxy terminus was expressed correctly in these mutants. While the anti-peptide antibody reacted specifically with ICP27 in Western blot analysis of HSV-1-infected cell lysates (P. J. Vaughan and R. M. Sandri-Goldin, submitted for publication), sufficient quantities of ICP27 were not produced in transfected cells to be detected by Western blot analysis. To circumvent this difficulty, we expressed the carboxy-terminal half of H7 and H17 in the bacterial fusion vector pATH3 (80). Each mutant was cloned from the *Sal*I site, which precedes the site of the deletions (Fig. 3; Table 1), to the *Sst*I site at the end of the ICP27 fragment. We also cloned two other mutants, S1B and N2, to compare the fusion protein profiles of these two insertion mutants with those of the deletions and that of the wild-type ICP27-Trp fusion protein.

The Coomassie-stained SDS-polyacrylamide gel of C600 cell extracts containing wild-type, N2, H7, and S1B constructs showed the presence of a strong band corresponding to the fusion protein for each in the indoleacrylic acid-induced samples (Fig. 7). The apparent molecular mass of the wild-type ICP27-Trp fusion protein was approximately 63 kilodaltons. This is in good agreement with the predicted molecular mass of the fusion protein, which contains 250 amino acids of ICP27 from residues 262 to 512 and 332 residues of the *E. coli* Trp protein (7, 80). The S1B fusion protein migrated with an apparent molecular mass which was similar to that of the wild-type protein. This mutant contains an insertion of four residues between amino acids 406 and 407 (Table 1). The N2 protein, however, migrated with an apparent molecular mass approximately 4 kilodaltons greater than that of the wild-type fusion protein. The N2 mutant contains an insertion of four amino acids between residues 459 and 460. This insertion resulted in the addition of two arginine residues to a relatively uncharged region of the protein. This may account for the increased mobility of

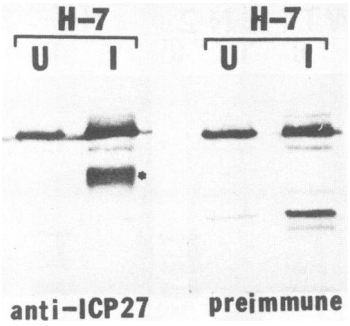

FIG. 8. Western blot analysis of the H7-Trp fusion protein reacted with ICP27 anti-peptide antibody specific for the carboxy terminus. C600 cells containing the H7-*trp* fusion plasmid were either left uninduced (U) or induced with indoleacrylic acid (I). Whole-cell lysates were divided in half, and each aliquot was fractionated on an SDS-polyacrylamide gel. The protein were transferred to nitrocellulose by electroblotting. Each of the two identical blots was treated with a different antiserum. The blot on the left was treated with an ICP27 antiserum which was raised against a synthetic peptide comprising the terminal 12 amino acids of the protein as predicted from the DNA sequence (see Materials and Methods). The blot on the right was treated with rabbit preimmune serum.

the fusion protein. The H7 fusion protein migrated with an apparent molecular mass of around 55 kilodaltons. This is consistent with a deletion of a total of 62 residues resulting from the initial deletion of 66 residues and an insertion of four amino acids. The identity of each ICP27 fusion peptide was confirmed by Western blot analysis with the ICP27 fusion protein antibody raised against the ICP27-Trp fusion protein produced by ICP27-pATH3 (data not shown).

The H7 fusion protein was analyzed on a Western blot by using the anti-peptide antibody which is specific for the terminal 12 amino acids of ICP27 (see Materials and Methods). The H7 fusion protein in the induced sample reacted with the anti-peptide antibody, whereas there was no specific reactivity seen in the uninduced sample or in an identical blot treated with preimmune serum (Fig. 8). A similar result was found for the H17 fusion protein. A peptide of the predicted molecular mass (about 48 kilodaltons) reacted with the ICP27 anti-peptide antibody (data not shown). These results confirm the sequence analysis and demonstrate that the carboxy-terminal region of the H7 and H17 peptides is expressed in frame. This was further tested by introducing an additional insertion into each mutant to cause a frame shift after the deletion. Mutants H7 and H17 were digested with *Eco*RI because a 12-bp *Eco*RI linker was originally inserted into each mutant (Table 1). Following the digestion, the four-base overhang was filled in with Klenow and the plasmids were religated. This resulted in the addition of 4 bp to each mutant so that the carboxy-terminal region was now out of frame beyond the site of the deletion. Each of these mutants, termed H7-2 and H17-2, was tested in transfections with ICP4 and ICP0 with pTK-CAT as the target. Both mutants lost the ability to repress activation of pTK-CAT (data not shown).

These results further support the hypothesis that the carboxy-terminal domain is essential for the repressor activity of ICP27. Both deletion mutants, H7 and H17, retained the repressor function when the terminal 62 amino acids remained intact. On the other hand, mutants which have insertions in the region from amino acids 434 to 505 have lost the repressor activity (Table 1). To further test the importance of this region, a mutant was constructed in which the carboxy-terminal domain was deleted. Mutant B-S was constructed by digesting the wild-type ICP27 plasmid with *Bal*II, which cuts at nucleotide 1712, and *Ssp*I, which cuts at nucleotide 1925, and then religating the plasmid DNA. The resulting plasmid, B-S, contained a 213-bp deletion and encoded a protein with a net loss of 71 amino acids from residues 434 to 505 encompassing the carboxy-terminal domain. The results of transfection assays with pTK-CAT as the target showed that CAT activity was induced 47 (±9)-fold when plasmids encoding ICP4 and ICP0 were included in the transfections, and this level was reduced to 3 (±1.2)-fold when the wild-type ICP27 plasmid was added. In transfections with deletion mutant B-S, the level of induction of pTK-CAT seen was 42 (±11)-fold, showing that the deletion mutant B-S lost the ability to repress pTK-CAT activation by ICP4 and ICP0. The values reported are mean values from six separate transfections.

The carboxy-terminal region of ICP27 encodes several cysteine and histidine residues which are similar in configuration to zinc finger metal-binding domains found in a number of transcriptional regulatory proteins (3, 4, 21, 28). The consensus sequence of Berg (3, 4) has the structure $C\text{-}X_{2\text{–}4}\text{-}C\text{-}X_{2\text{–}15}\text{-}C/H\text{-}X_{2\text{–}4}\text{-}C/H$, whereas the potential metal-binding domain of ICP27 is $C_{(483)}\text{-}X_4\text{-}C_{(488)}\text{-}X_{13}\text{-}H_{(502)}\text{-}X_5\text{-}C_{(508)}$. The positions of the amino acids of ICP27 involved in the putative zinc finger region are given in parentheses. While the spacing between the second H-C pair is one amino acid more than that found in the consensus sequence (3, 4), this spacing is identical to that found in one of the two zinc fingers in the glucocorticoid receptor (21, 28). Furthermore, insertion of four amino acids between the H at position 502 and the C at 508 resulted in loss of repressor activity in mutant S18 (Fig. 4), suggesting that this spacing is important. In addition, the structural integrity of this region appears to be critical because although only the terminal 62 amino acids were required for repression in deletion mutant H7, insertions into the region up to 50 nucleotides upstream of this domain also resulted in loss of repressor activity in mutant B7. It is possible that the inserted amino acids at this site caused a change in the tertiary structure of the protein around the putative metal-binding domain, rendering it nonfunctional. This region is also important for the activation function of the protein, since mutants in the carboxy-terminal domain were also defective in that activity (Table 1).

A summary of the positions of the insertion and deletion mutants in the ICP27 gene is presented in Fig. 9. Also shown is the region which contains the putative metal-binding domain in the carboxy terminus. The regions of the protein involved in repression and activation are indicated also.

DISCUSSION

We previously showed that the HSV-1 α protein ICP27 could act as a *trans*-repressor or as a *trans*-activator in combination with two other α proteins, ICP4 and ICP0 (74). The specificity of action depended on the target plasmid (74). The HSV-1 *trans*-activator ICP4 can also act as a repressor, as can the E1A *trans*-activator protein of adenovirus (6, 11, 24, 25, 59, 67, 84). These proteins were shown by mutational analysis to contain different domains which were important for each function (12, 54, 60). To identify the regions of the ICP27 protein which are involved in the repressor and activator functions, we constructed a series of in-frame insertion and deletion mutants at different sites throughout the ICP27 gene. These mutants were assayed for the ability

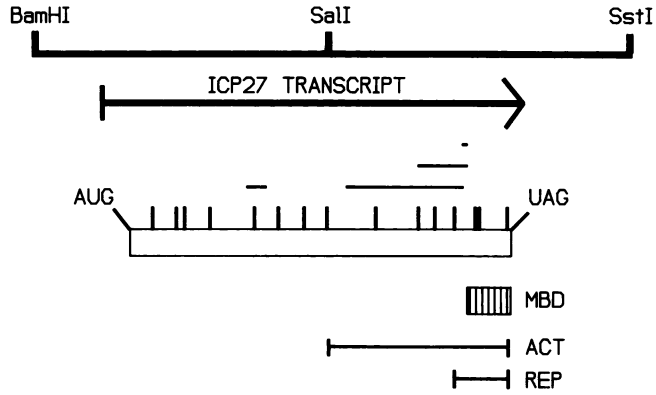

FIG. 9. Summary of the positions of the ICP27 insertion and deletion mutations. The *Bam*HI-*Sst*I fragment which contains the ICP27 gene is shown along with the position of the transcript and the coding sequence. The position of each of the insertion mutations is indicated by a vertical line above the coding sequence. The deletion mutations are shown as horizontal lines above the coding sequence, and the length of the line indicates the extent of the deletion. The region encompassing the putative metal-binding domain is shown by the box labeled MBD. The region identified as important for the activation function is shown (ACT), as is the region found to be important for repression (REP).

to repress or activate two different target genes in the presence of ICP4 and ICP0 (Fig. 4). As with the wild-type ICP27 plasmid, when the mutant plasmids were added to transfections in the absence of ICP4 and ICP0, no effect was seen on the expression of the target plasmids (data not shown). However, in the presence of ICP4 and ICP0, mutants containing insertions between residues 262 and 406 were unable to activate pVP5-CAT, although these mutants retained the ability to repress pTK-CAT (Table 1; Fig. 4). Mutants which contained insertions between residues 434 and 505 lost both repressor and activator functions. The hydrophilic amino-terminal half of ICP27 did not appear to be essential for either activity, since mutants in this region retained both activities.

The carboxy-terminal half of ICP27, which is required for the activation function, is relatively hydrophobic and does not appear to encode regions which are similar in nature to those which have been defined for some other *trans*-activator proteins. For example, there are no "leucine zippers" (44), glutamine-rich regions (9), or highly acidic stretches (47). In fact, the most acidic region of the protein is in the amino terminus in the first 70 residues. This region does not appear to play a role in the activation or repression functions of ICP27, because mutant N9M, which contains an insertion of eight amino acids, including two arginines, thus changing the acidic nature of the protein around the insertion, was wild type with respect to both activities (Fig. 4). The one region of ICP27 which contains a domain which is similar to regions found in other transcription proteins is in the carboxy-terminal 60 to 70 amino acids. This region contains a number of cysteine and histidine residues which resemble metal-binding domains (3, 4). A putative C-C-H-C zinc finger region, similar to one of the zinc fingers in the glucocorticoid receptor DNA-binding domain (21, 28), can be seen between residues 483 and 508. Mutants in and around this region were defective in the repressor and activator functions (Table 1). On the other hand, mutants in which this region remained intact retained the repressor function. These results suggest that this potential metal-binding domain functions in either DNA binding or protein interactions, as has been shown for some other *trans*-activating factors (4, 21, 28). The notion that this is a zinc-binding domain is further supported by the finding that ICP27 is quantitatively retained on a metal-chelating column in the presence of zinc but not in its absence, showing that ICP27 can bind zinc (Vaughan and Sandri-Goldin, submitted). The carboxy-terminal region is the only one in the protein which has a potential zinc finger motif. Furthermore, ICP27 does appear to have DNA-binding activity, since we found that ICP27 bound to a DNA-agarose column (Vaughan and Sandri-Goldin, submitted), substantiating a previous report by Hay and Hay (30). We did not determine whether ICP27 binds to specific sequences.

It is also possible that ICP27 interacts directly with ICP4 and ICP0 in addition to any DNA-binding activity which it may have. This is suggested by the fact that ICP27 had little activity on its own in transfection experiments but required the presence of ICP4 and ICP0. Furthermore, Su and Knipe (79) reported a mobility shift in ICP4 from cells transfected with an ICP27 plasmid compared with cells that express ICP4 alone, which also suggests that these two proteins interact in some way. We found no differences in the expression of ICP4 in the presence of ICP27 either quantitatively or qualitatively in transfections with a target which was repressed compared with one which was activated (Fig. 1). This suggests that while ICP4 and ICP27 may interact, ICP27 does not switch from acting as a repressor to an activator by altering ICP4 expression.

Another suggestion that ICP4 and ICP27 interact comes from the analysis of the proteins eluted from metal-chelating columns to investigate whether ICP27 binds zinc (Vaughan and Sandri-Goldin, submitted). While most of the ICP4 from HSV-1-infected cell lysates was not retained by these columns, a small amount was retained which eluted with ICP27. ICP4 does not contain regions which are similar to zinc-binding domains (52), and therefore it is possible that the protein which was retained was bound in some way to ICP27. If protein interactions occur between ICP27 and ICP4 and ICP0, it may involve domains which are structurally similar rather than domains with apparent sequence homology. This is because ICP4 and ICP0 do not share very much sequence homology and because ICP27 can also act functionally with IEP from PRV (Fig. 2). It is also possible that ICP27 interacts with a cellular factor, which in turn interacts with these other *trans*-activators.

The mutants described in this report are being recombined into the viral genome to compare their in vivo phenotypes with those which we defined in the transfection assays. Furthermore, mutant peptides isolated from infections with these mutants will be analyzed by metal chelate chromatography and DNA-binding assays to correlate these properties of ICP27 with the regions which have been shown to be important for activation and repression.

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