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# Amino Acid Substitution Mutations in the Herpes Simplex Virus ICP27 Protein Define an Essential Gene Regulation Function

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**ICP27 is an essential herpes simplex virus type 1 (HSV-1) α protein that is required for the transition from the β to the γ phase of infection. To identify functional regions of ICP27, we constructed 16 plasmids that contain nucleotide substitution mutations in the ICP27 gene. The mutations created *Xho*I restriction sites, altered one or two codons, and were spaced at semiregular intervals throughout the coding region. Three mutations completely inactivated an essential function of ICP27, as demonstrated by the inability of the transfected plasmids to complement the growth of an HSV-1 ICP27 deletion mutant. These mutations, M11, M15, and M16, mapped in the carboxyl-terminal one-third of ICP27 at residues 340 and 341, 465 and 466, and 488, respectively. In cotransfection assays, all three defective-plasmid mutants retained the transrepression function of ICP27 but were defective at transactivation. To define the lytic functions that are mediated by the transactivation activity of ICP27, we engineered HSV-1 recombinants containing the M11, M15, or M16 mutation. All three viral mutants failed to grow in Vero cells and possessed similar phenotypes. The viral mutants replicated their DNA similarly to the wild-type virus but showed several defects in viral gene expression. These were a failure to down-regulate α and β genes at late times after infection and an inability to induce certain γ-2 genes. Our results demonstrate that the transactivation function of ICP27 (as it is defined in cotransfection assays) mediates an essential gene regulation function during the HSV-1 infection. This activity is not required for ICP27-dependent enhancement of viral DNA replication. Our work supports and extends previous studies which suggest that ICP27 carries out two distinct regulatory activities during the HSV-1 infection.**

Herpes simplex virus type 1 (HSV-1), a widespread human pathogen, is the prototypic member of the alphaherpesvirus family (40). In vivo, HSV-1 can replicate lytically in a variety of cell types and can establish latent infections in neurons. In many cultured cells, HSV-1 undergoes a rapid and efficient productive infection, allowing both biochemical and genetic analyses of the lytic cycle. This characteristic distinguishes HSV-1 from most of the other human herpesviruses, which in general replicate poorly in cell culture. HSV-1 is therefore a valuable model for understanding some of the basic replication pathways of human and other herpesviruses.

The events of the lytic HSV-1 infection occur in a temporally ordered and highly regulated fashion (reviewed in reference 40). The DNA genome of HSV-1 encodes more than 70 genes and is transcribed in the infected cell nucleus by the host RNA polymerase II. The HSV-1 genes have been classified into three groups depending on the temporal order of, and functional requirements for, their expression (21). The α, or immediate-early, genes are the first class of genes to be expressed. Transcription of these genes can occur in the absence of new viral protein synthesis and is stimulated by the action of the virion transactivator VP16 (also called Vmw65, α-TIF, or ICP25). The function of the α proteins appears to be largely regulatory, as discussed in detail below. The β, or delayed-early, genes are the next to be expressed. Transcription of the β genes requires the action of the α protein ICP4 (for infected-cell protein). For the most part, the protein products of the β genes are involved directly or indirectly in viral DNA replication. Viral DNA synthesis begins soon after expression of the β proteins and stimulates the expression of the last class of viral genes, the γ, or late, genes. The γ genes have been subdivided on the following basis: γ-1, or leaky-late, genes are expressed at low levels in the absence of DNA replication, while γ-2, or true-late, genes are not expressed at readily detectable levels in the absence of replication. Most of the γ genes encode components of the HSV-1 virion.

Four of the five α proteins (ICP4, ICP0, ICP22, and ICP27) are localized to the nuclei of infected cells (1) and have been demonstrated to perform regulatory functions during the lytic infection. ICP4, the best characterized of these proteins, has an essential role in the transcriptional activation of β and γ genes (8, 16, 34, 50). ICP4 possesses a sequence-specific DNA binding activity that appears to be required for its transcriptional-activation function (23, 31, 33, 45).

Our laboratory is interested in the regulatory functions of the α protein ICP27. ICP27 is a 512-residue phosphoprotein that migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular mass of 63 kDa (21, 27, 41, 52). It is essential for HSV-1 growth since viral ICP27 null mutants are completely defective for lytic replication (26, 36). Of interest is the fact that herpesviruses from each of the three major classes (alpha-, beta-, and gammaherpesviruses) encode proteins with recognizable homology to ICP27 (2, 7, 32, 49, 53). Such conservation across the herpesvirus kingdom suggests that ICP27 carries out one or more functions that are fundamental to herpesvirus replication.

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Phenotypic studies of HSV-1 mutants bearing defined lesions in the ICP27 gene have been useful in ascribing specific functions to ICP27. HSV-1 mutants which are null for ICP27 because of large gene deletions are 5- to 10-fold deficient in viral DNA replication, are unable to down-regulate α or β genes, and are unable to efficiently induce γ genes (26, 36). These regulatory effects are separable, at least in part, as demonstrated by the analysis of amino (N)-terminus- and carboxyl (C)-terminus-specific ICP27 mutants. The *d*1-2 mutant, which encodes an ICP27 molecule lacking an acidic region near its N terminus, is deficient in DNA replication but does not show severe defects in gene expression (38). In contrast, the *n*504R mutant, which encodes an ICP27 molecule with a short truncation at its C terminus, is not deficient in DNA replication but does show a severe restriction to the expression of many of its γ genes and is unable to down-regulate α and β genes (36, 38). These studies have supported a model (38) in which ICP27 carries out two distinct regulatory activities that together contribute to the β-to-γ transition. One activity appears to enhance the efficiency of viral DNA replication, while a second activity appears to mediate the induction of certain γ genes and the repression of α and β genes.

ICP27 has also been demonstrated to mediate regulatory activities in uninfected cells. When expressed from a transfected plasmid, ICP27 can either activate or repress the expression of certain cotransfected reporter genes (4, 9, 35, 43, 48). Positive and negative regulation of cotransfected reporter genes by ICP27 (hereafter referred to as the transactivation and transrepression activities, respectively, of ICP27) are genetically separable functions, as demonstrated by the analyses of various ICP27 mutants (20, 29, 39). Furthermore, these regulatory activities may be mediated at the level of mRNA processing. Evidence for this comes from the identification of the *cis*-acting signals in the reporter genes which determine whether, and how, they are regulated by ICP27 (6, 42). The *cis*-acting DNA elements which confer transactivation by ICP27 are sequences bearing polyadenylation signals (6, 42). In contrast, transrepression of reporter genes by ICP27 has been correlated with the presence of introns in the reporter gene (42).

An obvious but as yet unanswered question is how the transactivation and transrepression activities, defined in cotransfection assays, relate to the functions of ICP27 during the lytic infection. In this study, we have identified three substitution mutations in the C-terminal one-third of ICP27 which completely abrogate its transactivation activity. Although two of these mutations map in a C-terminal region that has previously been implicated in transrepression (20, 29), all three mutations leave ICP27's transrepression activity fully intact. To define the role of the transactivation activity in the lytic infection, the three substitution mutations were engineered into recombinant viruses. We show that all three viral mutants possess a common phenotype, characterized by wild-type (WT) levels of viral DNA replication but aberrant gene expression. Our results demonstrate that, during infection, the transactivation activity of ICP27 mediates an essential gene regulation function. This activity does not appear to be required for ICP27's ability to enhance viral DNA replication.

## MATERIALS AND METHODS

**Construction of plasmids.** The parental plasmid for these studies was pM27, which contains a 2.4-kb *Bam*HI-*Sst*I HSV-1 fragment obtained from the plasmid pBS27 (38) cloned in the *Bam*HI-*Sst*I sites of the vector pSELECT-Sal$^-$. This fragment contains the intact ICP27 gene, including its promoter and mRNA 3′-end cleavage and polyadenylation signals. pSELECT-Sal$^-$ is a derivative of pSELECT (Promega) in which the *Sal*I site was destroyed. This was accomplished by digesting pSELECT with *Sal*I, filling in the 3′ recessed ends with the large fragment of *Escherichia coli* DNA polymerase I, religating the blunt ends with T4 DNA ligase, and obtaining a plasmid clone after transformation of *E. coli*.

The construction of plasmids containing the M1 and M2 mutations, pBSpm12 and pBSpm64, was described previously (38). To construct pM1 and pM2, the HSV-1 inserts from pBSpm12a/b and pBSpm64a/b were cloned as *Bam*HI-*Sst*I fragments into pM27 in place of the WT ICP27 gene insert. Plasmids pM3 through pM16 were constructed from pM27 by oligonucleotide-directed mutagenesis with a commercially available kit (Altered Sites; Promega). For each plasmid mutant, two independent plasmid isolates, designated *a* and *b*, were obtained. The structure of each mutant plasmid was initially confirmed by restriction enzyme digestion to ensure that the expected *Xho*I restriction site had been introduced. The identity of each *a* derivative was then confirmed by sequencing the DNA through the region mutagenized. To accomplish this, relevant restriction fragments were subcloned into bacteriophage M13 vectors. Single-stranded phage DNA was prepared and sequenced at the University of Alberta Biochemistry Department Core DNA facility by using an Applied Biosystems model 373A DNA sequencing system. The corresponding fragments of pM27 were also sequenced to identify naturally occurring nucleotide differences between strain KOS1.1, which was the strain used in our experiments, and strain 17, for which the DNA sequence has been published (27). This analysis revealed three noteworthy points regarding the engineered mutants. First, as a result of the oligonucleotide-directed mutagenesis, mutant M5 contained a strain 17-specific codon 155 (GCT in strain 17 and GCC in KOS1.1, both encoding alanine). Second, the strain 17 and KOS1.1 sequences differ at codon 173, which was one of the codons mutagenized in mutant M6 (CCT in strain 17 and CCG in KOS1.1, both encoding proline; CTC in M6, encoding leucine). Third, strains 17 and KOS1.1 differ at codon 196 (GGC in strain 17 and GGA in KOS1.1, both encoding glycine). Although mutant M7 contained the expected substitution mutations at codons 200 and 201, it contained a KOS1.1-specific codon 196. This alteration was unexpected, since codon 196 was encompassed by the strain 17-specific mutagenic oligonucleotide, but it may have occurred as a result of mismatch repair in *E. coli*.

**Cells, viruses, and infections.** All infections and transfections were carried out in Vero cells (African green monkey kidney cells), obtained from the American Type Culture Collection, Rockville, Md., or in V27 cells, a line derived from Vero cells after stable transfection with the ICP27 gene (36). The cells were propagated in Dulbecco modified Eagle medium which contained 10% heat-inactivated fetal calf serum (GIBCO). HSV-1 strain KOS1.1 (22), originally provided by M. Levine (University of Michigan, Ann Arbor), was the WT strain of HSV-1 used in all experiments. Infections were carried out at a multiplicity of infection of 10 PFU per cell.

**Transfections and CAT assays.** Transfections were carried out by the calcium phosphate technique, as previously described (19, 35). For chloramphenicol acetyltransferase (CAT) assays, cell extracts were prepared after 2 days and relative CAT activities were measured by enzyme assay of an appropriate dilution of the cell extract (18, 35). The CAT activities were normalized according to the protein content of each extract, as determined with a Bio-Rad protein assay kit, which utilizes the Bradford assay (5). The amounts of cotransfected

TABLE 1. ICP27 gene mutations constructed by oligonucleotide-directed mutagenesis

| Mutation | Oligonucleotide[a] | ICP27 codon(s) altered | Amino acid substitution(s) |
|---|---|---|---|
| M1 | AATTGACCTCGAGCTGGACCTCTC | 12 | G to E |
| M2 | CGATACTCGAGGCCGCTCGCC | 64 | D to E |
| M3 | GTCGCCGGGCCTCGAGGCGCGAC | 109 | G to E |
| M4 | GCCCACGGCGTCCCTCGAGGGCAGGCTGGGC | 138, 139 | RG to LE |
| M5 | ACCATCGGCAGCCTCGAGACCACCGCGACC | 153, 154 | PG to LE |
| M6 | GGGGCGGGGTCCCTCGAGGTTGCGATTGGT | 173, 174 | PG to LE |
| M7 | GGCCTCCGGGTGGCTCGAGCTGCTCACTGCC | 200, 201 | PD to LE |
| M8 | GCGCCTTTCGCTCGAGGGCCGGGGCGCG | 235 | P to L |
| M9 | GCCCCCCAAAGGGCTCGAGCATGACCTGTGC | 277, 278 | HD to LE |
| M10 | CCGTCTGGTCTCGAGGTCAAAGGGCCCT | 305 | A to L |
| M11 | GGCGCAGCACGCACTCGAGCATGGCCTTGGC | 340, 341 | RD to LE |
| M12 | ATAATGGGGTCCTCGAGGCGCAGCGGCAG | 375, 376 | PQ to LE |
| M13 | TTCAGGTAGCACTCGAGAAAGGGCCGC | 399 | Q to E |
| M14 | GCTCGACGCGGTTCTCGAGCCTGGCCAG | 437 | A to E |
| M15 | CGCCATGCAGGCCTCGAGGAGGTAGAAATG | 465, 466 | PG to LE |
| M16 | GGCCGTCAACTCGAGGACACGACTCGAA | 488 | C to L |

[a] Underlined nucleotides denote changes from the published ICP27 gene sequence (27).

ICP27 plasmid DNA used in the transfection assays (8 μg for transactivation, 1 μg for transrepression) were based on our empirical determination of the amount of WT ICP27 plasmid which gives the highest level of transactivation and transrepression in the respective assays (37). The *d*27-1 virus complementation assays were carried out as described previously (38, 39).

**Isolation of recombinant viruses and marker rescue.** To transfer the mutant alleles into the HSV-1 genome, the M11, M15, and M16 mutations were first engineered into a plasmid, pPs27pd1 (39), which contains the ICP27 gene on a 6.0-kb HSV-1 insert. This step was done to increase the extent of HSV-1 sequences flanking the mutation, thereby increasing the expected frequency of in vivo homologous recombination. The 2.4-kb *Bam*HI-*Sst*I fragments of the pM11a, pM15a, and pM16a plasmids were cloned into pPs27pd1 in place of the corresponding WT fragment, and the resulting plasmids were designated pPsM11, pPsM15, and pPsM16, respectively.

The isolation of recombinant viruses containing the M11, M15, and M16 mutations was carried out by a marker transfer protocol (36). Briefly, pPsM11, pPsM15, and pPsM16 were cleaved with *Pst*I and individually cotransfected into V27 cells with infectious *d*27-lacZ1 DNA. The progeny from the cotransfection were plated in V27 cells in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Potential viral recombinants were isolated as clear plaques against a background of parental blue plaques. Each isolate was plaque purified three times in V27 cells, and small stocks were prepared in V27 cells. The stocks were used to infect cultures of V27 cells, and viral DNA was prepared by the method of Gao and Knipe (13). The viral DNAs were subjected to Southern blotting analysis to confirm the expected genomic structure of each mutant. Southern blotting and hybridizations were carried out by standard procedures (25).

To carry out marker rescue of the M11 mutant, infectious M11 viral DNA (2 μg) was mixed with 20 M equivalents of purified WT restriction fragments obtained from plasmid clones and transfected into V27 cells. Positive control fragments included a 4.1-kb *Sal*I-*Pst*I fragment and a 0.7-kb *Sal*I-*Ssp*I fragment, both of which encompassed the C-terminal half of the ICP27 gene. The viral progeny were harvested after 5 days, and titers were determined on Vero and V27 cells to ascertain the fraction of progeny which could form plaques on Vero cells. Both positive control fragments rescued the M11 mutation (≥10% of the WT growth phenotype), whereas no rescue (<0.002% of the WT growth phenotype) was observed in the absence of added restriction fragments or with a 1.9-kb *Pst*I-*Sal*I fragment which encompassed the N-terminal half of the ICP27 gene. Two marker-rescued isolates, M11r1 and M11r2, were obtained after two rounds of plaque purification in Vero cells. M11r1 was obtained from a marker rescue with the *Sal*I-*Ssp*I fragment, and M11r2 was obtained with the *Sal*I-*Pst*I fragment. As an additional control in the analysis of the rescued viruses, a pool of unrescued M11 progeny from the marker rescue transfection were grown in V27 cells to obtain a high-titered stock.

**Analysis of mutant viruses.** The analysis of viral DNA replication levels in HSV-1-infected cells was carried out as previously described (38). The isolation of cytoplasmic RNA and Northern (RNA) blot analysis were also performed as described previously (38). The probes for Northern analysis were the plasmids pE/3583 (ICP8 gene probe) (14) and pEcoRI-BamHI-I-I (gC gene probe) (12). The plasmid DNA probes were prepared by $^{32}P$ labeling by using the random-primer labeling technique (3).

For analysis of protein expression, infected cells were labeled for 1 or 6 h with 10 to 20 μCi of [$^{35}S$]methionine-cysteine per ml (EXPRE$^{35}$S$^{35}$S; DuPont) in methionine-free medium. Infected-cell proteins were harvested and subjected to SDS-gel electrophoresis in 9.25% polyacrylamide gels as described previously (15, 24). Immunoblotting of infected cell proteins was performed as described previously (39). To detect VP16, nitrocellulose filters were treated with a 1:10,000 dilution of SW8, a rabbit antiserum raised against a synthetic peptide antigen corresponding to residues 475 to 488 of VP16 (51). The SW8 antiserum was a generous gift of Steve Weinheimer, Bristol-Myers Squibb. Antigen detection was carried out by using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection system (Amersham).

## RESULTS

**Construction and characterization of plasmids encoding mutant ICP27 molecules.** We used oligonucleotide-directed mutagenesis to construct a set of 16 plasmids that contained nucleotide substitution mutations at sites throughout the ICP27 coding region (Table 1). Each mutation was designed to create a unique *Xho*I restriction enzyme site, and in every case

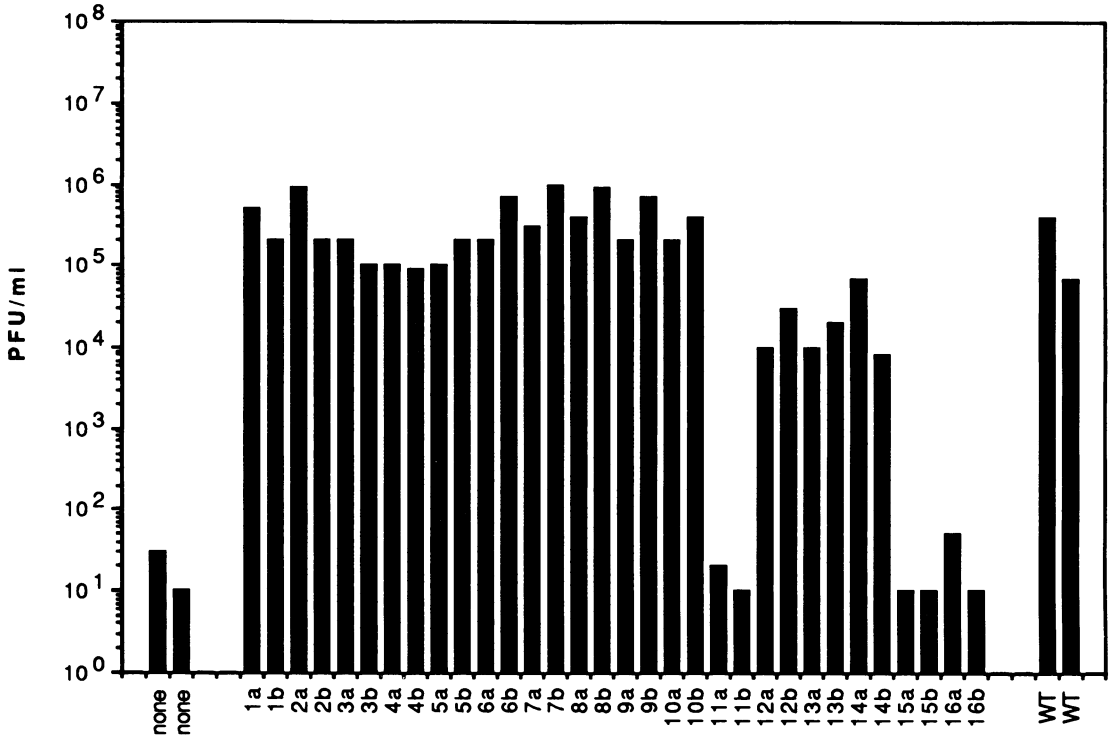


FIG. 1. Complementation of *d*27-1 growth by mutant ICP27 plasmids. Two identical cultures of Vero cells ($2 \times 10^6$ cells) were transfected with carrier DNA only (labeled none, at left) or with 8 μg of WT ICP27 plasmid pM27 (labeled WT, at right). Single cultures of Vero cells were transfected with 8 μg each of two isolates (*a* and *b*) of ICP27 mutant plasmids pM1 through pM16 (labeled 1a through 16b). One day after transfection, the cultures were infected with the HSV-1 ICP27 deletion mutant *d*27-1 and incubated for a further 24 h at 37°C. Virus was released from the cultures (10-ml total volume) by three cycles of freeze-thawing and titrated by plaque assay on V27 cells.

the nucleotide alterations resulted in one or two amino acid substitutions in the encoded ICP27 protein. All the *Xho*I recognition sites were introduced into the same reading frame, a feature that was intended to facilitate the future construction of in-frame deletion mutations in the ICP27 coding region.

To determine whether any of the substitution mutations affected an essential function of ICP27, we asked whether the plasmid-encoded proteins could complement the growth of an HSV-ICP27 deletion mutant, *d*27-1 (36). Vero cells were transfected with carrier DNA only or with WT or mutant ICP27 plasmids. For each mutant, two independently derived plasmid isolates, designated *a* and *b*, were tested. One day after transfection, the cultures were infected with *d*27-1 and incubated for an additional day. The yield of infectious virus in the cultures was then determined by plaque assay on V27 cells, derivatives of Vero cells which possess a stably transfected copy of the ICP27 gene and fully complement the growth of *d*27-1 and other HSV-1 ICP27 mutants (36). The results of this experiment are shown in Fig. 1. The plasmid mutants appeared to fall into three categories. The mutants pM1 through pM10 were as efficient as the WT plasmid in complementing the growth of *d*27-1. The mutants pM12, pM13, and pM14 were moderately defective for ICP27 function, exhibiting an approximately 10-fold decrease in complementation activity. These mutants were not studied further. In contrast, the pM11, pM15, and pM16 mutants exhibited either very slight or no ability to complement the growth of *d*27-1. The M11, M15, and M16 mutations therefore inactivate an essential function of ICP27.

In transfection assays, ICP27 can either transactivate or transrepress the expression of cotransfected reporter genes (4, 9, 35, 43, 48). Since the virus complementation experiment demonstrated that the M11, M15, and M16 mutants are defective in an essential lytic function, it was of interest to characterize the effect of these mutations on the transactivation and transrepression activities. First, we asked whether the three plasmid mutants were able to transactivate gene expression. The reporter gene used was gBCAT(−175), a construct previously shown to be responsive to ICP27 transactivation (35). The gBCAT(−175) plasmid contains the HSV-1 glycoprotein B promoter driving expression of the CAT gene. In addition, this gene contains a 3′ polyadenylation sequence which has been shown to confer ICP27 transactivation (42). Vero cells were transfected with pgBCAT(−175), with or without the WT or mutant ICP27 plasmids. Two days later, the cells were harvested and tested for CAT activity (Fig. 2A). As expected, cotransfection with the WT plasmid significantly induced CAT expression. All three mutants, however, were unable to stimulate the expression of gBCAT(−175). We conclude that the M11, M15, and M16 mutations disrupt the transactivation activity of ICP27.

We next tested the ability of the mutants to transrepress a cotransfected reporter gene. Although transrepression of reporter genes can be mediated by ICP27 alone (4, 42), the transrepression assay is often carried out in the presence of the HSV-1 transactivating protein ICP0 and/or ICP4 (43, 48), which greatly stimulates many HSV-1 promoters. In this case, ICP27 can fully block the stimulation of certain reporter genes by ICP4 and/or ICP0. The reporter gene we used was 8CAT, a construct which contains the HSV-1 ICP8 promoter driving

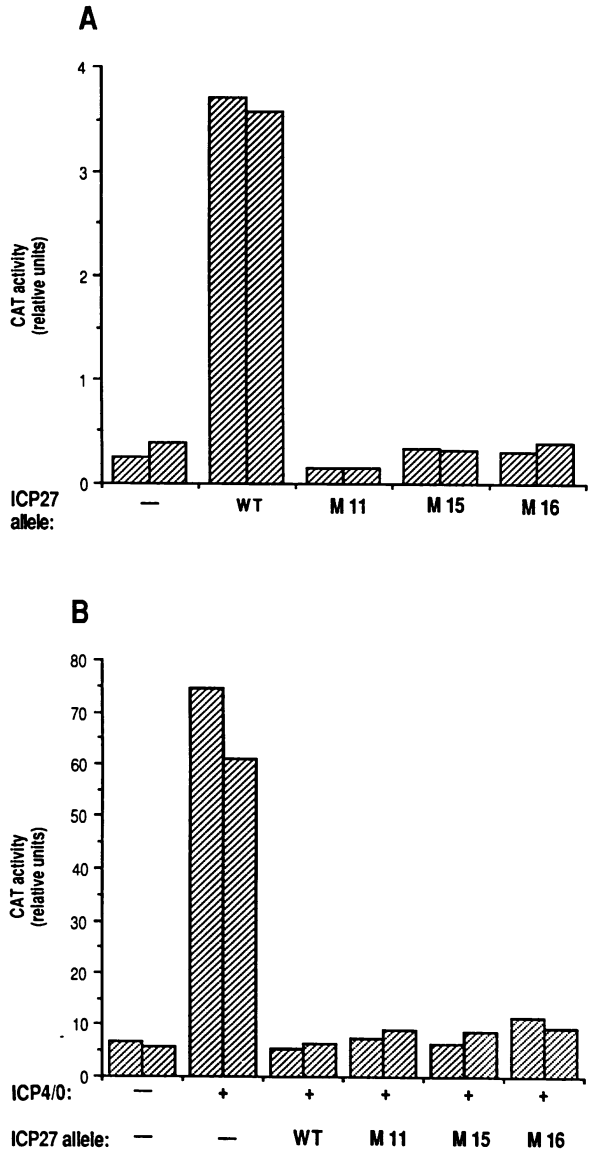


FIG. 2. Ability of the M11, M15, and M16 plasmids to regulate the expression of cotransfected reporter genes. WT or mutant plasmids were tested in transfection assays for the transactivation (A) or transrepression (B) functions of ICP27. (A) Transactivation assay. Vero cell cultures were transfected in duplicate with 8 μg of pgB-CAT(−175) plus, where indicated, 8 μg of WT or mutant ICP27 plasmids (−, no addition). The cells were collected after 2 days, and relative CAT activities were determined. Duplicate samples are shown as side-by-side bars. (B) Transrepression assay. Vero cell cultures were transfected in duplicate with 6 μg of p8CAT and, where indicated (+), 1 μg of pSG1 (17), encoding ICP4 and ICP0. Where indicated, 1 μg of the WT or mutant ICP27 plasmids was also added (−, no addition). Analysis and representation of CAT activities are as described in the legend to panel A.

expression of the CAT gene (48). This gene also contains a downstream intron derived from the simian virus 40 small t antigen gene. The presence of this intron has been correlated with the transrepression effect (42). The p8CAT plasmid was transfected alone, with a plasmid expressing ICP4 and ICP0 (the ICP4/0 plasmid), or with the ICP4/0 plasmid plus plasmids expressing either the WT or mutant ICP27 genes. As shown in Fig. 2B, cotransfection of the ICP4/0 plasmid stimulated expression of 8CAT, and inclusion of a plasmid encoding WT ICP27 was able to block this effect. All three mutant plasmids showed a repression activity that was equivalent to that of the WT plasmid. We conclude that the M11, M15, and M16 mutations do not significantly affect the transrepression activity of ICP27.

**Construction of viral mutants containing the M11, M15, and M16 mutations.** The experiments described above demonstrated that M11, M15, and M16 mutations specifically affect the transactivation activity of ICP27. Therefore, the phenotype of viral recombinants bearing the M11, M15, and M16 mutations should allow insight into the lytic functions that are mediated by this activity. We used a marker transfer protocol (36) to introduce the M11, M15, and M16 mutations into the HSV-1 genome. Since our complementation analysis indicated that these mutations inactivated an essential function (Fig. 1), potential HSV-1 recombinants were isolated in ICP27-complementing V27 cells.

To ensure that the intended mutations had been successfully introduced into the recombinant viruses, the mutant genomes were analyzed by Southern blotting (Fig. 3). In this analysis, we also included DNAs from WT strain KOS1.1 as well as *d*27-lacZ1, the recipient genome used in the marker transfer, which has an insertion of the *E. coli lacZ* gene in the ICP27 gene (36). The viral DNAs were digested with *Pst*I and hybridized with the WT 6.0-kb *Pst*I fragment that contains the ICP27 gene (Fig. 3A and B). As expected, the *d*27-lacZ1 genome, because of its *lacZ* insertion, yielded an 8.3-kb *Pst*I fragment (Fig. 3C, lane 1), while the WT fragment was 6.0 kb (lane 2). The M11, M15, and M16 genomes all yielded ICP27 gene *Pst*I fragments that comigrated with the WT fragment (Fig. 3C, lanes 3 to 5), demonstrating that the *d*27-lacZ1 gene had been replaced by alleles that were of the WT size. As expected, all of the viral genomes also yielded a common but less strongly hybridizing fragment which was derived from the opposite long repeated region-long unique region ($R_L$-$U_L$) junction (Fig. 3A). When the DNAs were digested with *Pst*I and *Xho*I (Fig. 3C, lanes 6 to 9), the M11, M15, and M16 genomes yielded two strongly hybridizing bands, demonstrating the presence of the introduced *Xho*I sites. Furthermore, these fragments were of the expected sizes (Fig. 3B) and comigrated with the *Pst*I-*Xho*I fragments from the donor plasmids used in the marker transfer (data not shown). These results demonstrate that the M11, M15, and M16 substitution mutations were successfully introduced into the recombinant viruses.

To see whether the M11, M15, and M16 mutants were defective for growth, we carried out a virus plaque assay. Although the mutants efficiently formed plaques on V27 cells, all three mutant virus stocks were unable to form plaques on Vero cells at any dilution tested (data not shown). Therefore, as expected, the M11, M15, and M16 mutations are lethal. To more quantitatively assess the replication defects of the mutants, we carried out a single-cycle virus yield experiment. Parallel cultures of Vero or V27 cells were infected at a multiplicity of infection of 10 with either the substitution mutants, the ICP27 null mutant *d*27-1, or the WT strain KOS1.1 and incubated for 24 h. Virus yield was determined by plaque assay on V27 cells. The results of this experiment (Table 2) indicated that the M11, M15, and M16 mutants were severely restricted for growth in Vero cells, yielding less than 0.002 PFU per infected cell. The very low yields observed for the substitution mutants were at least 5 orders of magnitude less than the WT virus yield in Vero cells. However, the yields

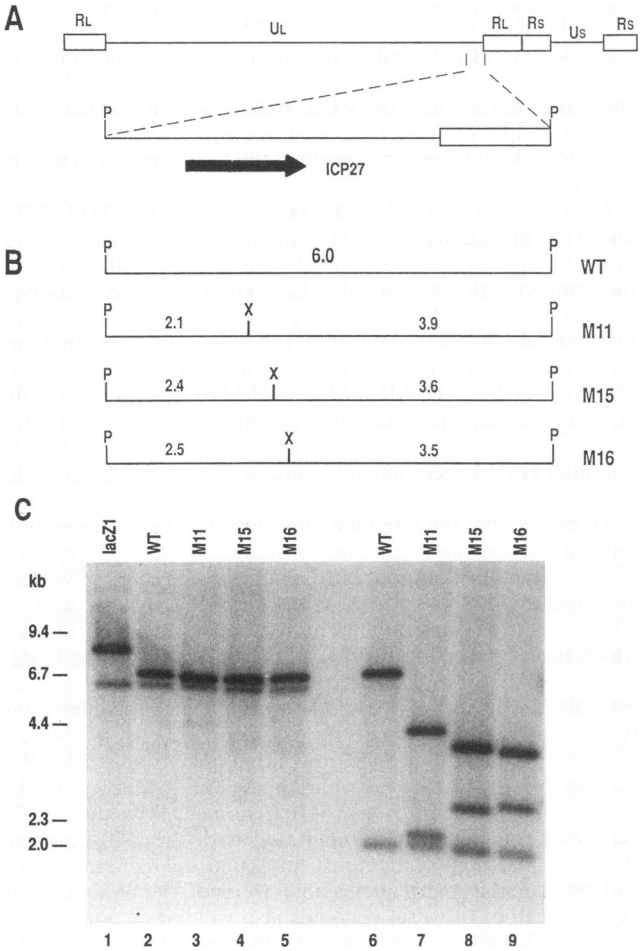


FIG. 3. Introduction of M11, M15, and M16 mutations into recombinant HSV-1 genomes. (A) Representation of the HSV-1 genome, including the region encoding ICP27. The drawing at the top illustrates the prototype arrangement of the HSV-1 genome, while the expanded drawing below shows the 6.0-kb *Pst*I (P) restriction fragment which contains the ICP27 gene. The solid lines denote long and short unique ($U_L$ and $U_S$) regions of the viral genome, and the open bars represent long and short repeated regions ($R_L$ and $R_S$). The arrow denotes the ICP27 coding region. (B) Structure of the ICP27-encoding *Pst*I restriction fragment from WT or mutant genomes. Restriction sites are indicated (P, *Pst*I; X, *Xho*I), as well as the sizes of restriction fragments in kilobase pairs. (C) Southern blotting analysis of mutant viral genomes. Viral DNAs from *d*27-lacZ1 (lacZ1) (lane 1), WT KOS1.1 (WT) (lanes 2 and 6), M11 (lanes 3 and 7), M15, (lanes 4 and 8), and M16 (lanes 5 and 9) were digested with *Pst*I alone (lanes 1 to 5) or with both *Pst*I and *Xho*I (lanes 6 to 9) and subjected to Southern blotting. The filter was probed with $^{32}$P-labeled DNA from a plasmid containing the WT 6.0-kb *Pst*I restriction fragment shown in panel B. Shown at the left are the positions of DNA size standards. Note that for each digestion, all viral DNAs yield a common and more weakly hybridizing fragment which is derived from the opposite $R_L$-$U_L$ junction.

of the substitution mutants were measurably higher (10- to 40-fold higher) than the yield observed for the null mutant *d*27-1. All three substitution mutants, as well as *d*27-1, yielded near WT levels of progeny virus when the viral infections were carried out in V27 cells. This result demonstrates that the replication defect of the M11, M15, and M16 mutants can be efficiently complemented by expression of the WT ICP27 protein from V27 cells.

TABLE 2. Growth properties of HSV-1 ICP27 mutants

| Virus | Virus yield (PFU/cell)[a] in cell line: | |
|---|---|---|
| | Vero | V27 |
| *d*27-1 | <0.000045 | 80 |
| M11 | 0.0019 | 36 |
| M15 | 0.00043 | 33 |
| M16 | 0.0014 | 36 |
| KOS1.1 | 190 | 100 |

[a] Vero cells ($2.2 \times 10^6$) were infected at a multiplicity of infection of 10 PFU per cell. The same inocula were used to infect parallel cultures of $2.5 \times 10^6$ V27 cells. The cultures were harvested at 24 hpi, and virus yield was determined by plaque assay on V27 cells.

**Phenotypic characterization of the M11, M15, and M16 viral mutants.** We next carried out experiments to identify which aspects of viral replication were altered in Vero cells infected with the M11, M15, and M16 mutants. Previous work has implicated ICP27 in both the enhancement of viral DNA synthesis and the regulation of gene expression during the late phase of infection (26, 29, 36, 38, 41). Therefore, we carried out experiments to examine both of these processes.

First, we characterized the ability of each mutant to replicate its DNA. Vero cells were mock infected or infected with the WT virus or ICP27 mutants. As controls, we included an ICP27 null mutant, *d*27-1, and an ICP27 nonsense mutant, *n*504R. The *n*504R mutant, which encodes an ICP27 molecule lacking its 12 C-terminal residues, shows the WT level of DNA synthesis but shows altered late-gene expression (36). The cultures were labeled with [$^3$H]thymidine from 6 to 12 h postinfection (hpi), at which time total DNA was purified from each culture. Equal amounts of DNA were digested with the restriction enzymes *Eco*RI and *Xba*I and electrophoresed on an agarose gel. The labeled DNAs were visualized by fluorography (Fig. 4). As expected, the *d*27-1-infected culture showed

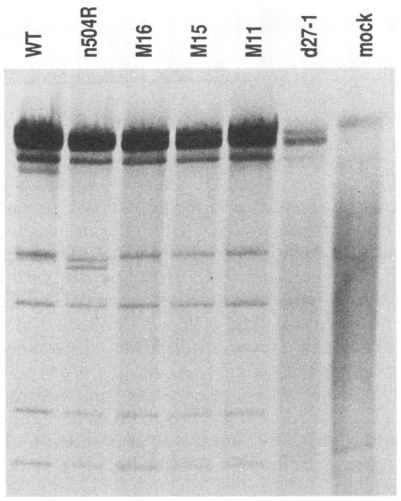


FIG. 4. Viral DNA replication in ICP27 mutant-infected cells. Vero cells were mock infected or infected with the viruses indicated at the top. The cells were labeled with [$^3$H]thymidine from 6 to 12 hpi. Total DNA was then purified, and equal amounts were digested with *Eco*RI plus *Xba*I and electrophoresed on a 0.9% agarose gel. The gel was processed for fluorography, dried, and exposed to X-ray film. The *n*504R genome contains an *Xba*I site at the 3′ end of the ICP27 gene (36) and therefore gives rise to a novel *Eco*RI-*Xba*I restriction fragment.

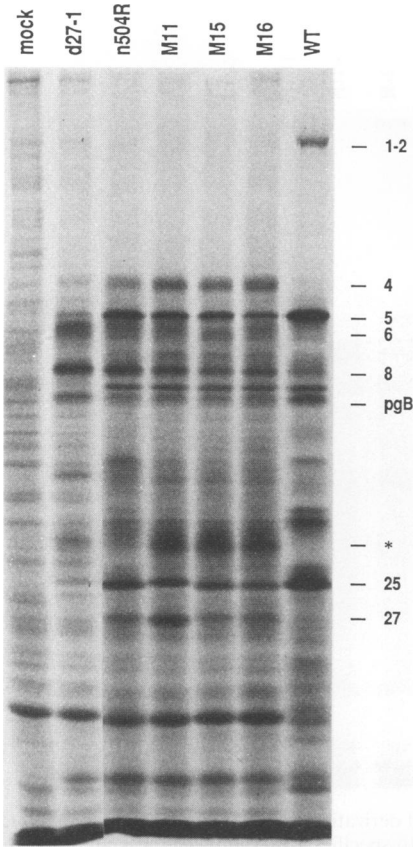


FIG. 5. Protein expression in ICP27 mutant-infected cells. Vero cells were mock infected or infected with the viruses indicated at the top. The cells were labeled with [$^{35}$S]methionine-cysteine from 6 to 12 hpi and then harvested. Equal fractions of each cell lysate were analyzed by SDS-PAGE and autoradiography. Shown at the right are the positions of several known HSV-1 proteins (ICP designations are indicated). An unknown protein of ~80 kDa which accumulates in M11-, M15-, and M16-infected cells is indicated by an asterisk.

low levels of DNA replication, consistent with the known role of ICP27 in the enhancement of viral DNA synthesis, and the *n*504R culture showed the WT level of DNA synthesis. All three substitution mutants also showed WT levels of viral DNA replication. Therefore, the M11, M15, and M16 mutations do not appear to affect the ability of ICP27 to enhance viral DNA replication.

We next examined viral late gene expression in cells infected with the M11, M15, and M16 mutants. First, we examined the production of viral proteins during the mid-to-late phase of infection. As before, the *d*27-1 and *n*504R viruses were included in the analysis. Infected Vero cells were labeled with [$^{35}$S]methionine-cysteine from 6 to 12 hpi, and the labeled proteins were analyzed by SDS-PAGE and autoradiography. The results of this experiment (Fig. 5) demonstrated that the M11, M15, and M16 mutants all express very similar patterns of proteins at late times after infection. This pattern was characterized by enhanced expression, compared with the WT infection, of α proteins (including ICP4 and the ICP27 polypeptide itself) and, to a lesser extent, of β proteins (in particular, ICP8). Of interest was the finding that the three substitution mutants, and to a lesser extent *n*504R, appeared to express higher levels of ICP4 from 6 to 12 hpi than did the null mutant, *d*27-1, which was previously shown to overexpress ICP4 relative to the WT virus (36). The other common alteration exhibited by the M11, M15, and M16 mutants was a deficiency in the expression of several γ proteins. Proteins of the γ-1 kinetic class, such as ICP5 and ICP25, were slightly reduced in abundance, whereas at least one γ-2 protein, ICP1-2 (30), was not detectable.

The pattern of gene expression exhibited by the M11, M15, and M16 mutants from 6 to 12 hpi was very similar to that of the previously characterized *n*504R mutant. However, there were some significant differences. In particular, we noted that all three substitution mutants, but not *n*504R, expressed very high levels of an ~80-kDa protein (indicated by an asterisk in Fig. 5). The identity of this protein is unknown but is currently under investigation.

Although the patterns of late proteins expressed by the three substitution mutants were quite similar to each other, M11 differed from M15 and M16 with respect to two proteins. First, the ICP25 protein expressed in M11-infected cells had a slightly slower electrophoretic mobility than it did in all the other infections. This was intriguing, since the ICP25 protein (also known as VP16, Vmw65, and α-TIF) is an important regulatory and structural protein, and ICP27 is known to have an effect on the electrophoretic mobility of at least one other viral protein, ICP4 (29, 35, 48). The identity of this protein as ICP25 (VP16) was confirmed by immunoblotting with a VP16-specific antiserum (Fig. 6A). The second difference between the M11 protein pattern and that of M15 and M16 concerned the ICP27 protein itself. Although all three substitution mutants overexpressed ICP27, M11-infected cells expressed somewhat higher levels of ICP27 from 6 to 12 hpi than did either M15- or M16-infected cells.

To see whether the two differences described above were due to the M11 mutation, a marker rescue analysis was carried out. Viruses showing WT growth in Vero cells were generated by marker rescue of the M11 mutation with WT ICP27 gene restriction fragments derived from cloned plasmid DNAs (see Materials and Methods for details). Two independently rescued viruses, designated M11r1 and M11r2, were isolated. We then carried out a protein-labeling experiment to compare the protein expression patterns at late times after infection in M11-, M11r1-, and M11r2-infected cells. The infected Vero cells were labeled with [$^{35}$S]methionine-cysteine from 7 to 8 hpi and analyzed by SDS-PAGE (Fig. 6B). The M11r1 and M11r2 viruses were nearly indistinguishable from the WT virus in their patterns of late viral protein synthesis, demonstrating that the major alterations in gene expression observed in M11-infected cells result from the ICP27 gene mutation in M11. This included the effect on expression of ICP27 itself, as the M11r1- and M11r2-infected cells showed normal down-regulation of ICP27 expression at late times after infection (Fig. 6B and data not shown).

However, careful inspection of the autoradiogram shown in Fig. 6B and those from a repeat experiment revealed that M11r1- and M11r2-infected cells expressed an ICP25 protein that migrated with the same slow electrophoretic mobility as the M11 ICP25 protein. We conclude that the alteration in ICP25 (VP16) electrophoretic mobility seen in the M11 mutant is not due to the M11 mutation but is due to an unrelated secondary mutation, most likely in the ICP25 gene itself. It is important to note that the M11r1 and M11r2 rescuants show WT growth in Vero cells and express a pattern of late viral proteins that is extremely similar to the WT pattern. Therefore, the secondary mutation in M11 does not appear to contribute in a significant way to its mutant phenotype.

Our protein expression experiments demonstrated that at

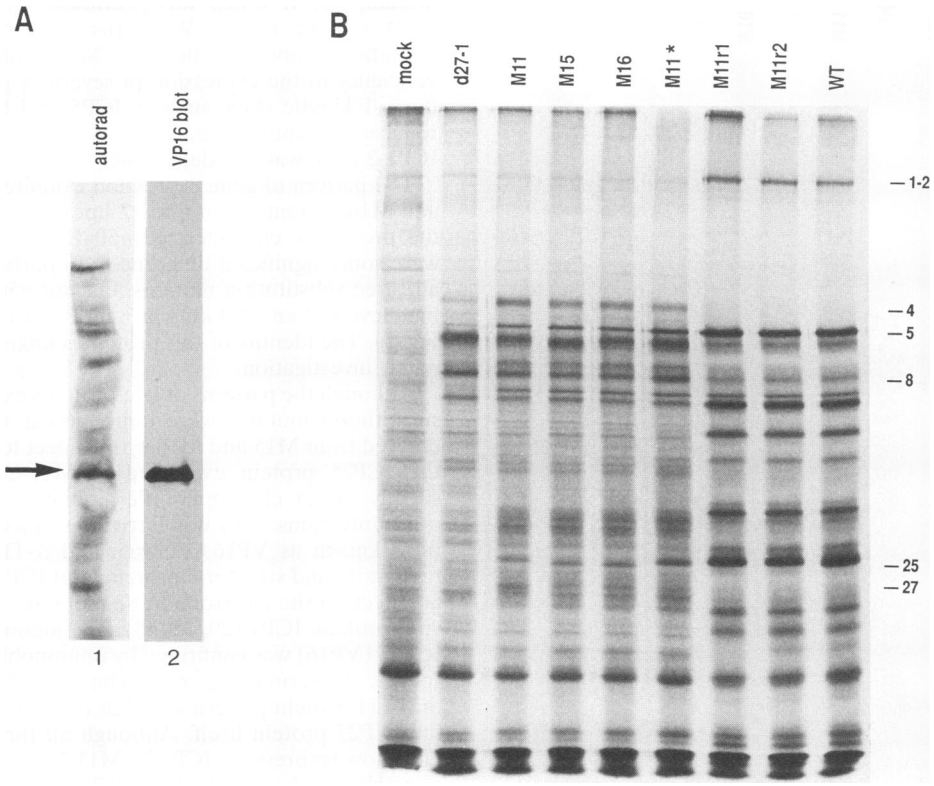


FIG. 6. Analysis of protein expression in cells infected with M11 or marker-rescued derivatives of M11. (A) VP16 immunoblot. A $^{35}$S-labeled protein sample from WT-infected cells was subjected to immunoblotting with a VP16-specific antiserum. Lane 1 is an autoradiograph of the immunoblot filter (overnight exposure) showing all of the labeled proteins, including the protein we designate ICP25 (arrow). Lane 2 shows detection of VP16 (ICP25) on the same filter by immunoblotting with an enhanced chemiluminescence detection system (10-min exposure). (B) Protein expression in infected cells. Vero cells were mock infected or infected with the viruses indicated at the top. The infected cells were labeled with [$^{35}$S]methionine-cysteine from 7 to 8 hpi and analyzed by SDS-PAGE and autoradiography. M11* refers to a pool of unrescued M11 progeny from the same marker rescue transfection that gave rise to M11r1 and M11r2. The positions of several known HSV-1 proteins are indicated (ICP designations) on the right.

least one γ-2 protein, ICP1-2, was not induced to detectable extents in the M11, M15, and M16 infections, despite WT levels of viral DNA synthesis. We have previously shown that the expression of another γ-2 gene, that encoding the glycoprotein C (gC), is also dependent upon ICP27 (36). In the case of the gC gene, we demonstrated that ICP27 is required for the appearance of mature gC mRNA. Furthermore, we found that gC mRNA is not induced in *n*504R infections, indicating that an intact ICP27 C terminus is required for gC mRNA induction. To see whether gC mRNA was expressed in M11, M15, and M16 infections, we carried out Northern blotting experiments. Vero cells were mock infected or infected with WT virus or ICP27 mutants. Cytoplasmic RNA was isolated from infected Vero cells at 10 hpi, and equal amounts were electrophoresed on denaturing agarose gels and transferred to nylon filters. Hybridization was first carried out with a probe specific for a β gene, that encoding ICP8 (Fig. 7A). Consistent with the protein analyses, the M11, M15, and M16 mutants expressed higher levels of ICP8 mRNA at late times after infection than did the WT virus. However, when hybridization was carried out with the γ-2 gC probe, we found that the M11, M15, and M16 mutants, like *n*504R and *d*27-1, were unable to efficiently induce expression of gC mRNA. Comparison of different autoradiographic exposures of this blot demonstrated that the level of gC mRNA in the M11, M15, and M16 infections was

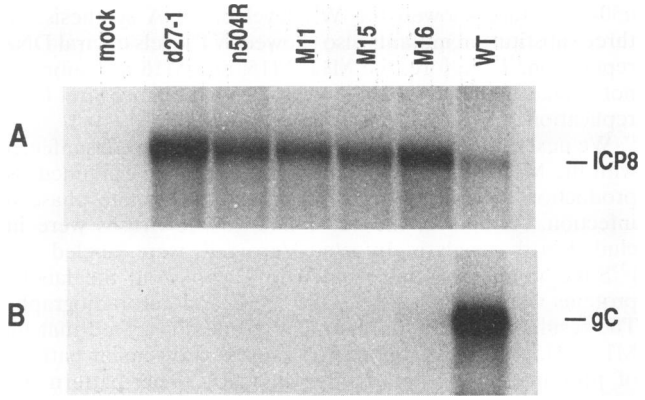


FIG. 7. Defective accumulation of γ-2 gC mRNA in ICP27 mutant-infected cells. Vero cells were mock infected or infected with the viruses indicated at the top. Cytoplasmic RNA was isolated at 10 hpi, and equal amounts (10 μg) were subjected to Northern blot analyses using $^{32}$P-labeled probes specific for ICP8 mRNA (A) or gC mRNA (B).

reduced more than 30-fold compared with the level found in the WT infection.

## DISCUSSION

**Isolation of plasmids with amino acid substitution mutations which inactivate ICP27.** Several of the regulatory proteins encoded by DNA viruses replicating in the cell nucleus have been shown to be composed of multiple and in some cases independent functional domains. Well-characterized examples include the adenovirus E1A proteins (11) and the simian virus 40 large T antigen (10). Such multifunctional proteins may be a common feature of these viruses, which have limited coding capacity but must interact with, and in some cases modify, multiple components of the infected cell nucleus. The existing data suggest that ICP27 is also a multifunctional regulatory protein, affecting both HSV-1 DNA replication and gene expression.

In the present study, we have engineered a set of 16 mutant plasmids that contain nucleotide substitution mutations spaced at semiregular intervals throughout the ICP27 coding region. These mutants were constructed with two aims in mind. The first was to create a set of plasmid reagents that could be used in the future to construct additional plasmids containing in-frame deletions. The 16 mutations were designed so that all create *Xho*I restriction sites in a common reading frame. Therefore, any two plasmids can be used to engineer a third plasmid which contains an in-frame deletion between the two parental *Xho*I sites. We have already used this strategy to construct and characterize an HSV-1 mutant which bears an in-frame deletion near the N terminus of ICP27 (38). More recently, we have isolated several additional recombinant viruses possessing in-frame deletion mutations in the ICP27 gene (37).

The second aim in constructing the nucleotide substitution mutants was to gain further insight into the structure and function of ICP27. We reasoned that amino acid substitution mutations in ICP27 might be interesting for a number of reasons. First, although temperature-sensitive (*ts*) mutants mutated in ICP27 have been obtained, no nonconditional lethal mutants resulting from substitution mutations have been isolated. Unlike *ts* mutants, substitution mutants of the type we have generated are not constrained to function under any conditions and might therefore display phenotypes not exhibited by conditional mutants. Second, amino acid substitution mutants are less likely to disrupt ICP27 tertiary structure than previously isolated insertion (20), deletion (29, 38), or nonsense (29, 36) mutants and therefore might lead to more specific phenotypic effects. Indeed, as discussed below, the M15 mutation is associated with a different phenotype in cotransfection assays than is a linker insertion mutation mapping at the same site. Finally, recombinant viruses bearing substitution mutations in ICP27 may be useful in the future for isolating second-site reversion mutants. Such revertants can be extremely helpful in defining intramolecular protein interactions and in some cases can display altered phenotypes which are informative (44).

Of the 16 mutations we engineered, only the mutations in the C-terminal half of ICP27 affected its essential functions. Our results are reminiscent of those of Hardwicke et al. (20), who found that linker insertion mutations in the C-terminal, but not N-terminal, half of ICP27 affected its gene regulatory functions in transfected cells. It thus appears that the N-terminal half of ICP27 is more tolerant of amino acid substitutions or short insertions than is the C-terminal half. However, the N-terminal half of ICP27 is required for ICP27's regulatory activities, because a viral mutant bearing an in-frame deletion near the N terminus of the ICP27 coding region fails to replicate in Vero cells (38). These observations underscore the importance of analyzing different types of mutations when studying protein structure and function.

**Location of the transrepression function of ICP27.** In cotransfection assays, ICP27 can mediate both gene transactivation and transrepression. A number of studies have been carried out with ICP27 plasmid mutants to map the regions of ICP27 which are involved in these two activities (20, 29, 39). While most of the data suggest that the C-terminal half of ICP27 is critical for transactivation, there are some apparent discrepancies concerning which regions are involved in transrepression. For example, on the basis of the phenotype of a number of linker insertion mutants, Hardwicke et al. (20) have proposed that the repression activity of ICP27 maps to the C-terminal 78 residues (residues 434 to 512). However, both we (39) and McMahan and Schaffer (29) have found that a truncated ICP27 molecule not containing the C-terminal 106 residues could mediate either complete (39) or partial (29) transrepression. These results suggest that the transrepression function maps to sequences N terminal to residue 406. Consistent with this idea, we recently found that deletion of an acidic region at the N terminus of ICP27 significantly decreased its transrepression activity (38).

The results of this study are relevant to the location of the transrepression region of ICP27. The M11, M15, and M16 plasmid mutations, which map at codons 340 and 341, 465 and 466, and 488, respectively, inactivate the transactivation function of ICP27, consistent with previous mutational analyses (20, 29, 39). However, the M11, M15, and M16 mutations do not affect transrepression. It is noteworthy that the M15 and M16 mutations fall in the C-terminal region where linker insertion mutations inactivate transrepression function (20). In fact, the M15 mutation maps at the same site as the linker insertion mutation S2 described by Hardwicke et al. (20), a mutation which causes a four-residue insertion between amino acids 465 and 466. Thus it would appear that substitution mutations in the C-terminal 78 residues can inactivate transactivation without affecting transrepression, whereas small insertions in the same region lead to inactivation of both activities. Recent analysis of the *ts* mutation in the HSV-1 ICP27 *ts* mutant *ts*LG4 is consistent with this idea. This mutation was recently identified as a point mutation which alters codon 480 (47). In transfection assays, the ICP27 allele of *ts*LG4 is defective at high temperature for transactivation but not transrepression (20).

Two hypotheses that would reconcile most of the existing data concerning the location of the transrepression region can be advanced. First, as suggested by McMahan and Schaffer (29), ICP27 might contain two regions which can mediate transrepression. A second explanation, which we raise here, is that certain types of mutations in the C-terminal 78 residues can interfere with a transrepression activity which maps elsewhere in the molecule. We postulate that mutations which have this effect are those that cause significant structural alterations to the C-terminal region, as would be expected for linker insertion mutations. In this model, the C-terminal region of ICP27 would not be required for transrepression but could have a modulatory role in some circumstances. Further mutational studies are required to test these and other hypotheses.

**The role of the transactivation function during HSV-1 infection.** We have previously presented evidence which suggests that ICP27 carries out two distinct functions during the HSV-1 infection which together result in the transition from the β to the γ phase (36, 38). We proposed that one activity

acts to enhance viral DNA replication approximately 10-fold, indirectly stimulating the expression of all γ genes. A second activity appears to directly affect gene expression, independent of DNA replication, resulting in the stimulation of certain γ-2 genes and the repression of α and β genes.

In transfection assays, ICP27 also carries out two regulatory activities. One activity acts to transactivate gene expression, while the other acts to transrepress gene expression. A critical question is how the transactivation and transrepression functions of ICP27, defined in cotransfection assays, relate to ICP27's activities in lytically infected cells. Previous studies of ICP27 deletion and nonsense mutations in the context of recombinant viruses have not been totally conclusive, since the viral mutants have had multiple and complex phenotypes, most often including defects in both viral DNA replication and viral gene regulation (29, 36).

The M11, M15, and M16 mutants are informative in this regard. These substitution mutations are less likely than previous mutations to disrupt ICP27 protein structure. In cotransfection studies, all three mutations disrupt the transactivation activity of ICP27 but have no demonstrable effect on transrepression. In the context of recombinant viruses, the three mutations all lead to a common phenotype which is characterized by several defects in viral gene expression. These include a failure to down-regulate α and β genes and an inability to induce several γ-2 genes. Therefore, during viral infection, the transactivation activity of ICP27 acts to mediate the essential gene regulation function which we previously attributed to ICP27 (38). The term transactivation may be a misnomer, because in infected cells this activity is required for both gene repression and activation. However, at present we do not know whether the gene repression mediated by ICP27 in infected cells is direct or indirect. It is possible that the direct transactivation of γ genes by ICP27 leads indirectly to the down-regulation of α and β genes.

It is important to note that the M11, M15, and M16 viral mutants replicate their DNA to WT levels. Therefore, the transactivation activity is not required for ICP27's ability to enhance viral DNA replication. Our work thus supports and extends previous studies which suggest that ICP27 carries out two distinct regulatory activities during the HSV-1 infection (36, 38).

In transfection assays, the transactivation activity of ICP27 correlates with the presence of certain polyadenylation signals in the reporter genes, suggesting that ICP27 regulates these genes at a posttranscriptional level (6, 42). By inference, then, the repression of α and β genes and the induction of γ-2 genes during HSV-1 infection may be mediated by posttranscriptional mechanisms which are dependent on polyadenylation signals. This hypothesis is consistent with the recent observation that ICP27 is required for the induction of LPF, a factor that selectively increases RNA 3′-end processing at an HSV late polyadenylation site (28). However, it should be noted that ICP27 has also been reported to affect the transcription rates of viral early and late genes (26, 46). Further experimentation is required to determine the mechanisms by which α, β, and γ genes are regulated by ICP27 in infected cells.

Our studies shed light on the role that the transactivation function of ICP27 plays during the lytic HSV-1 infection. However, it is not yet clear what role, if any, the transrepression activity plays. One interesting possibility is that the transrepression function is involved in ICP27's ability to stimulate viral DNA replication. However, at present it is not possible to carry out a genetic study analogous to the one described here. This is because, to date, no ICP27 plasmid mutants have been described which are fully competent for transactivation but are defective for transrepression. Furthermore, if the transrepression function depends in some way on the transactivation function, it may be impossible to generate such mutants. However, the M15 mutation may provide a strategy to gain insight into the transrepression phenomenon. On the basis of the results of Hardwicke et al. (20), we expect that a linker insertion derivative of M15 will be defective for both transactivation and transrepression. If so, we plan to generate an HSV-1 recombinant virus that bears this mutation and compare its phenotype with that of M15. This comparison should elucidate the role of the transrepression function of ICP27.

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