

The Acidic Amino-Terminal Region of Herpes Simplex Virus Type 1 Alpha Protein ICP27 Is Required for an Essential Lytic Function

STEPHEN A. RICE,^{1*} VIVIAN LAM,¹ AND DAVID M. KNIPE²

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7,¹ and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115²

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The herpes simplex virus type 1 (HSV-1) α protein ICP27 regulates the transition between the delayed-early and late phases of the viral infection. Previous genetic analyses have suggested that the important functional domains of ICP27 map to its carboxyl-terminal half. One striking feature of the primary sequence of ICP27, however, is an extremely acidic region near its amino terminus. To determine whether this region is required for ICP27 function, we deleted the sequences in the ICP27 gene which encode it (codons 12 through 63). In transient expression assays, the deletion mutant was unable to efficiently repress the expression of a cotransfected reporter gene or to efficiently complement the growth of *d27-1*, an HSV-1 ICP27 null mutant. These results suggested that the acidic region of ICP27 is involved in a regulatory function required for lytic growth. To test this possibility further, we introduced the mutant allele into the HSV-1 genome by marker transfer. Two independently derived isolates of the mutant virus, designated *d1-2a* and *d1-2b*, were recovered and analyzed. Both isolates were defective for growth in Vero cells, exhibiting a 100-fold reduction in virus yield compared with the wild-type infection. Vero cells infected with the *d1-2* isolates showed a three- to eightfold reduction in viral DNA replication, a moderate reduction in the expression of viral γ genes, and a delay in the repression of β genes. The phenotype of the *d1-2* isolates differs substantially from the phenotypes of previously isolated ICP27 mutants, which show much more severe defects in viral gene expression. Our results demonstrate that the amino-terminal half of ICP27 participates in its regulatory activities in both infected and transfected cells.

The lytic infection of a susceptible cell by herpes simplex virus type 1 (HSV-1) consists of a temporally regulated program of viral gene expression, viral DNA replication, and virion assembly (reviewed in reference 36). The HSV-1 genome, a linear molecule of double-stranded DNA, is approximately 152,000 bp in size and encodes over 70 proteins. Soon after the virus penetrates the cell, the viral genome enters the nucleus, where the viral genes are transcribed by the host RNA polymerase II. The first genes to be transcribed are called α (or immediate-early) genes. As discussed in more detail below, the α genes encode proteins which regulate the infectious process. One function of the α proteins is to induce the transcription of the β (or delayed-early) genes. The protein products of the β genes are largely enzymes involved in viral DNA synthesis. Soon after the expression of β proteins, viral DNA replication begins. The process of viral DNA replication is critical for the expression of the last set of viral genes, the γ (or late) genes. The γ genes for the most part encode proteins which are components of the virus particle or are involved in its assembly. The γ genes have been subdivided according to the extent to which their expression requires viral DNA replication; the expression of γ -1 (or leaky-late) genes is reduced but readily detectable in the absence of DNA replication, while the expression of γ -2 (or true-late) genes is more stringently dependent on DNA replication. Following γ gene expression, DNA-containing virions are assembled in the cell nucleus and then mature and exit the cell via a complex interaction with the host secretory apparatus.

Genetic studies have demonstrated that two of the five α proteins have regulatory roles which are essential for viral growth (7, 25, 29, 37). One of these, infected cell protein 4 (ICP4), is the major transcription-activating protein of HSV-1; it is required throughout infection for the transcription of the β and γ genes (14, 29, 47). The 175-kDa ICP4 polypeptide has an intrinsic sequence-specific DNA-binding activity (19), but it is not yet clear how this activity is involved in transcriptional activation (40, 41). The second essential α protein is the 63-kDa ICP27 polypeptide. As discussed below, ICP27 appears to mediate the transition between the β and γ phases of viral infection. Like ICP4, ICP27 is a phosphoprotein which accumulates in the cell nucleus (1, 20, 49), but its biochemical properties have not been characterized in great detail. Recently, it was reported that purified ICP27 can bind to zinc ions and can interact with single-stranded DNA (46).

The analysis of viral mutants containing temperature-sensitive mutations in the ICP27 gene first demonstrated that ICP27 has an essential regulatory role during infection (37). Subsequently, viral deletion mutants which do not express ICP27 or any fragment of it were isolated (25, 33). Such mutants are defective for growth in normal cells but can be efficiently propagated in ICP27-expressing cell lines. These mutants exhibit a 5- to 10-fold reduction in viral DNA replication, fail to repress β gene expression at late times, and show dramatically reduced levels of γ -1 and γ -2 gene expression. Several other ICP27 virus mutants, including five nonsense mutants and one in-frame deletion mutant, have been isolated (26, 33). One nonsense mutant, *n504R*, has a phenotype which is of particular interest (33). The *n504R* mutant encodes an ICP27 polypeptide in which the

* Corresponding author. Electronic mail address: steve_rice@darwin.biochem.ualberta.ca.

carboxyl (C)-terminal eight amino acid residues are replaced by four new residues (the wild-type [WT] protein consist of 512 residues). Cells infected with the *n504R* mutant exhibit defects in the expression of γ -2 genes but show normal levels of viral DNA replication and γ -1 gene expression. On the basis of the phenotype of the *n504R* mutant, we proposed that ICP27 carries out two functions which contribute to the β - γ transition, one which stimulates γ -1 gene expression and viral DNA replication and a second which induces γ -2 genes, independent of DNA replication (33).

Transient-expression experiments have also provided evidence for the regulatory activities of ICP27. ICP27 can either positively or negatively affect the expression of cotransfected reporter genes (3, 8, 32, 39, 44). Positive regulation of reporter genes in many cases has been shown to be a synergistic effect which requires a second herpesviral transactivator, either ICP4, ICP0 (another α -regulatory protein), or the pseudorabies virus major immediate-early protein. However, in some cases ICP27 can act in the absence of other transactivators to stimulate expression of reporter genes (5, 32, 38). Negative regulation of reporter genes is somewhat more complicated in that ICP27 usually has little effect by itself but appears to inhibit activation by herpesviral transactivators. The *cis*-acting elements in the reporter genes which mediate positive and negative regulation have not been definitively identified, but recent studies have indicated that mRNA processing signals are critical to the response of a reporter gene to regulation by ICP27. Specifically, 3' sequences involved in pre-mRNA cleavage and polyadenylation appear to mediate positive regulation by ICP27 (5, 38), while intron sequences appear necessary for transrepression (38). These studies suggest that ICP27 can regulate gene expression at a posttranscriptional level in transfected cells, possibly by affecting mRNA processing.

Several mutational studies have been carried out with the aim of mapping the functional domains of ICP27 which are involved in activating and repressing gene expression in transfected cells (17, 26, 35). Although these studies have demonstrated that the positive and negative regulatory activities of ICP27 can be distinguished genetically, there are still several unresolved questions about where the functional regions of ICP27 map. For example, both Hardwicke et al. (17) and McMahan and Schaffer (26) concluded that the regions important for repression and activation mapped to C-terminal half of the ICP27 molecule. However, we found that the N-terminal half of the ICP27 molecule exhibited a low but measurable transactivation activity (35). In addition, we found that a truncated ICP27 molecule, entirely lacking the C-terminal region defined by Hardwicke et al. as the repression domain, still showed a WT ability to transrepress gene expression (35). McMahan and Schaffer found that a very similar truncation mutant exhibited partial repression activity (26). These apparent discrepancies might be explained if the different types of mutations that have been analyzed lead to different regulatory effects, or if ICP27 contains multiple transactivation or repression domains.

One feature of ICP27 which is striking is the high proportion of acidic amino acid residues in its amino (N)-terminal region. Twenty-five of the first 64 residues in ICP27 consist of either glutamic or aspartic acid. In addition, ICP27, a known phosphoprotein (49), contains nine serine residues in this region which are potential phosphate acceptors and hence might contribute to the overall negative charge. Similar highly acidic regions are found in many transcription factors, including the HSV-1 VP16 protein, and are thought to stimulate transcription via an interaction with one or more

components of the RNA polymerase II transcription complex (43). It was therefore of interest to examine whether the acidic N terminus of ICP27 plays a role in its function. We specifically deleted the sequences encoding this region from the ICP27 gene and assayed the mutant gene in transfection assays and in the context of the viral genome. Our results indicate that the N-terminal acidic region contributes to the regulatory activities of ICP27 in both transfected and infected cells.

MATERIALS AND METHODS

Cells, viruses, and infections. All infections and transfections were carried out in Vero 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 (33). The cells were propagated in Dulbecco modified Eagle medium plus 10% supplemented newborn calf serum (Calf Supreme; GIBCO). HSV-1 strain KOS1.1, 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 10 PFU per cell.

Construction of plasmids. Plasmid pBS27, containing the intact ICP27 gene, was constructed by cloning a 2.4-kb HSV-1 *Bam*HI-*Sst*I fragment derived from plasmid pBH27 (32) into the *Bam*HI and *Sst*I sites of pGEM3Zf-(Promega Biotec, Madison, Wis.). Plasmid pSVSK4, which expresses ICP4 under the control of the simian virus 40 (SV40) early-region promoter, was constructed in two steps. First, a 342-bp *Hind*III-*Pvu*II SV40 DNA fragment containing the SV40 enhancer and early-region promoter was cloned into pUC19. To accomplish this, the fragment was isolated from plasmid pSV2-CAT (15), the 3' recessed *Hind*III end was filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I, and the fragment was ligated into the *Xba*I site of pUC19 by using *Xba*I linkers. A clone, pSVE- α , was isolated which had the SV40 fragment oriented such that the SV40 promoter was directed toward the *Sal*I site of pUC19. Next, the ICP4 gene, derived from plasmid pSG1-EK1 (30), was cloned as a *Sal*I fragment into the *Sal*I site of pSVE- α . A plasmid, pSVSK4, was isolated which contained the *Sal*I fragment oriented such that the SV40 promoter was adjacent to the 5' end of the ICP4 gene. This plasmid expresses ICP4 after transfection into Vero cells, as judged by immunofluorescence and Western immunoblot analysis (31).

Construction of the deletion mutant plasmids pBSd1-2a and pBSd1-2b was done in two steps. First, oligonucleotide-directed mutagenesis was used to construct plasmid mutants pBSpm12 and pBSpm64, which contained point mutations in the ICP27 coding region. The mutations altered codons 12 and 64, respectively, by creating *Xho*I restriction sites. Mutagenesis was performed by the gapped heteroduplex technique (22, 27), using the oligonucleotides AATTG ACCTCGAGCTGGACCTCTC and CGATACTCGAGGC CGCTCGCC (underlined nucleotides denote alterations from the WT sequence), respectively, for the pBSpm12 and pBSpm64 mutants. For each plasmid mutant, two independently derived isolates were obtained and designated a and b. To construct the desired deletion, pBSpm12a and pBSpm64a plasmid DNAs were digested with *Eco*RI (which cuts at the 3' end of the HSV-1 insert) and *Xho*I. The large 3,650-bp *Eco*RI-*Xho*I fragment from pBSpm12a was ligated to the smaller 1,800-bp *Xho*I-*Eco*RI fragment from pBSpm64a, resulting in a plasmid, pBSd1-2a, which encoded an ICP27 gene in which codons 12 through 63 had been

deleted. An identical procedure was carried out with plasmids pBSpm12b and pBSpm64b, generating plasmid pBSd1-2b. The mutations introduced into pBSd1-2a and pBSd1-2b were confirmed by DNA sequencing. As expected, the manipulations resulted in a 156-bp deletion in the 5' coding region of the ICP27 gene, removing codons 12 through 63. In addition, the mutation altered codon 64 from aspartic to glutamic acid.

For transfer of the mutant alleles into the HSV-1 genome, the pBSd1-2a and pBSd1-2b mutations were engineered into a plasmid, pPs27pd1 (35), which contains the ICP27 gene on a 6.1-kb HSV-1 insert. This was done by cloning the 2.4-kb *Bam*HI-*Sst*I fragments of pBSd1-2a and pBSd1-2b into pPs27pd1 in place of the corresponding WT fragment. The resulting plasmids were designated pPsd1-2a and pPsd1-2b. 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.

Analysis of mutant plasmids. Transfections were carried out by the calcium phosphate technique as previously described (16, 32). 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 (32). The CAT activities were normalized according to the protein content of each extract. Western blotting was carried out as described previously (35), using a 1:300 dilution of H1113, a monoclonal antibody directed against ICP27 (1). The virus complementation assays were carried out as described previously (35) except that at 2 h postinfection (hpi), the cells were treated for 2 min with a glycine-saline solution (pH 3.0) as described by Cai et al. (4). This treatment significantly lowered the level of background unadsorbed virus without significantly affecting virus yields (34).

Isolation of mutant viruses. The isolation of ICP27 mutant viruses was carried out by a marker transfer protocol that we have previously described (33). Briefly, plasmids pPsd1-2a and pPsd1-2b were cleaved with *Pst*I and individually cotransfected into V27 cells with infectious *d27-lacZ*I DNA. The progeny from the cotransfection were plated in V27 cells in the presence of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. Potential viral recombinants were isolated as clear plaques against the background of parental blue plaques. To test whether the isolates encoded a deleted form of ICP27, individual clear plaques were used to infect 25-cm² flasks of V27 cells. The cultures were harvested when all the cells showed cytopathic effects. The resulting virus stocks were diluted 1:10 and used to inoculate cultures of Vero cells. Proteins were harvested at 8.5 hpi and subjected to Western blot analysis using the H1113 antibody. Several of the plaques gave rise to stocks which produced an immunoreactive protein of the expected size (approximately 52 kDa). Two such plaques, arising from marker transfers carried out with pPsd1-2a and pPsd1-2b DNAs, respectively, were plaque purified in V27 cells and designated *d1*-2a and *d1*-2b. Southern blot analysis of the viral DNAs confirmed that the *d1*-2a and *d1*-2b alleles had been successfully introduced into the recombinant viral genomes in place of the WT ICP27 gene (34).

Analysis of mutant viruses. To analyze viral DNA replication, 25-cm² culture flasks of Vero or V27 cells were mock infected or were infected with WT HSV-1 or various ICP27 mutants. At 9 hpi, the medium was replaced with 2 ml of medium containing 25 μ Ci of [³H]thymidine (New England Nuclear) per ml. At 12 hpi, the labeling medium was replaced with 3 ml of lysis buffer containing 10 mM Tris

hydrochloride (pH 8), 10 mM EDTA, 2% sodium dodecyl sulfate (SDS), and 100 μ g of proteinase K per ml. After overnight incubation at 37°C, sodium acetate was added to 0.3 M, and the lysates were extracted once with phenol-chloroform-isoamyl alcohol (24:24:1) and once with chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated after the addition of 2 volumes of ethanol and resuspended in 0.5 ml of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA (TE buffer). The samples were digested with 25 μ g of RNase A per ml for 30 min at 37°C, phenol-chloroform-isoamyl alcohol extracted as described above, and made 0.8 M in sodium chloride. An equal volume of 13% polyethylene glycol (average molecular weight, 8,000) was added. The samples were incubated on ice for 1 h and then centrifuged for 10 min at 13,000 \times *g*. The DNA pellets were washed with 70% ethanol, dried, and resuspended in TE buffer. DNA concentration were determined by measuring UV absorbance at 260 nm. Equal amounts of each DNA preparation (4 μ g) were digested with *Eco*RI and *Xba*I and electrophoresed on a 0.9% agarose gel. After electrophoresis, the gel was treated with a fluorography-enhancing agent (Entensify; DuPont Corp.), dried, and exposed to X-ray film at –70°C.

For analysis of protein synthesis, infected cells were pulse-labeled for 30 min with 15 μ Ci of [³⁵S]methionine-cysteine (Tran³⁵S-label; ICN Biomedicals) per ml. Harvesting of infected-cell proteins and SDS-polyacrylamide gel electrophoresis (PAGE) in 9.25% polyacrylamide gels were performed as described previously (13, 21).

Cytoplasmic RNA for Northern (RNA) blot analysis was prepared at 12 hpi by extraction of the infected cells with 0.5% Nonidet P-40 (24), followed by phenol-chloroform extraction of the supernatant and ethanol precipitation. The RNA was then resuspended in TE buffer, digested with RNase-free DNase (Boehringer Mannheim), phenol-chloroform extracted, and ethanol precipitated. Ten micrograms of each RNA sample was subjected to electrophoresis through denaturing formaldehyde-agarose gels and transferred to GeneScreen filters (DuPont). Hybridization of ³²P-labeled DNA probes, radiolabeled by the random primer extension method (2), was carried out overnight at 68°C in 6 \times SSC (1 \times SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–5 \times Denhardt solution (1 \times Denhardt solution is 0.2% [wt/vol] bovine serum albumin, Ficoll [molecular weight, 400,000], and polyvinyl pyrrolidone)–0.5% SDS–250 μ g of denatured salmon sperm DNA per ml. The filters were washed twice at 65°C in 2 \times SSC–0.1% SDS and then three times at 65°C in 0.2 \times SSC–0.1% SDS. The dried filters were then exposed to X-ray film. Plasmid DNAs were used for the hybridization probes. For the ICP8 (UL29) gene probe, plasmid pE/3583 (12) was used. Plasmid pBICP5 served as the ICP5 (UL19) gene probe. This plasmid was generated by cloning the *Bam*HI insert from the bacteriophage M13 clone, ICP5a (14), into the *Bam*HI site of pUC19. Plasmid pEcoRI-*Bam*HI-I-I (11) was used as the glycoprotein C (UL44) gene probe.

RESULTS

Analysis of plasmids encoding an ICP27 molecule lacking an acidic N-terminal region. To test the functional role of the acidic N-terminal region of ICP27, we used site-specific mutagenesis to alter an ICP27-encoding plasmid so that the sequences encoding this region were deleted. The parental plasmid was pBS27, which contains a 2.4-kb HSV-1 DNA insert encoding the intact ICP27 gene, including the 5' and 3' sequences needed for its expression in uninfected Vero cells

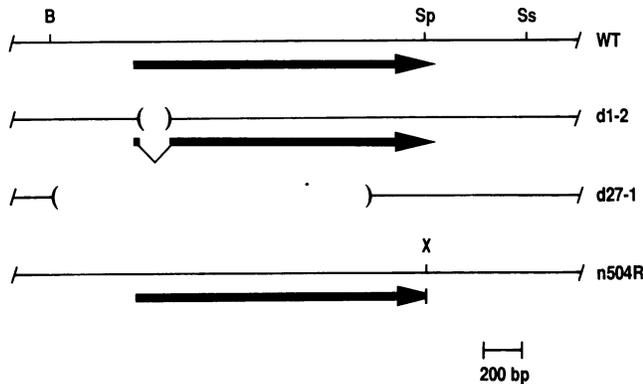


FIG. 1. Structure and coding potential of WT and mutant ICP27 genes. The horizontal lines represent HSV-1 DNA sequences contained in the plasmids and viruses used in this study. The parentheses indicate deletion of DNA sequences, while the letters denote relevant restriction enzyme sites (B, *Bam*HI; Sp, *Ssp*I; Ss, *Ssr*I; X, *Xba*I). The arrows below the lines represent the ICP27 polypeptides encoded by each allele. The *d1-2* polypeptide possesses an N-terminal in-frame deletion, while the *n504R* polypeptide (33) possesses a short C-terminal truncation due to the insertion of an *Xba*I-stop codon linker into the *Ssp*I site. The pBS series of plasmids used in this study carry inserts of the *Bam*HI-*Ssr*I fragment.

(32, 37). The mutant derivative, pBSd1-2, contained a deletion in the N-terminal coding region of the ICP27 gene which resulted in the removal of codons 12 through 63 (Fig. 1). Twenty of the 52 codons deleted encode negatively charged aspartic and glutamic residues, while 9 encode serine residues which are potential sites of phosphorylation. Two independently derived isolates of the mutant plasmid were recovered and designated pBSd1-2a and pBSd1-2b. These were analyzed separately in the experiments described below.

We first investigated whether the mutant ICP27 plasmids had alterations in the ability to transactivate gene expression in transfected cells. The reporter gene used was gBCAT (-175), a construct in which the HSV-1 glycoprotein B gene promoter drives expression of the CAT gene (32). We have previously shown that this gene is induced by ICP27, either alone or in concert with other herpesvirus transactivators (32). WT or mutant ICP27 plasmids were cotransfected into Vero cells with pgBCAT(-175), and 2 days later the cells were harvested and tested for CAT activity. As a negative control, we also transfected the mutant ICP27 plasmid pBH-59R (35), which encodes a truncated ICP27 molecule of 59 amino acid residues. This mutant has previously been shown to be defective for both the transactivation and transrepression functions of ICP27 (35). The results of the CAT assay are shown in Fig. 2A. As expected, the WT ICP27 plasmid showed a significant (seven- to eightfold) induction of CAT activity, whereas plasmid pBH59R was not able to transactivate expression of the reporter gene. Both isolates of plasmid pBSd1-2 showed transactivation levels that were comparable to that of the WT plasmid. These results demonstrate that the acidic N-terminal region is not required for the ability of ICP27 to transactivate the gBCAT(-175) reporter gene.

Next, we tested the ability of the mutant plasmids to transrepress gene expression in transfected cells. We examined whether the mutant plasmids could mediate the repression of an ICP4-transactivated reporter gene, 8-CAT, in which the HSV-1 ICP8 gene promoter drives CAT expres-

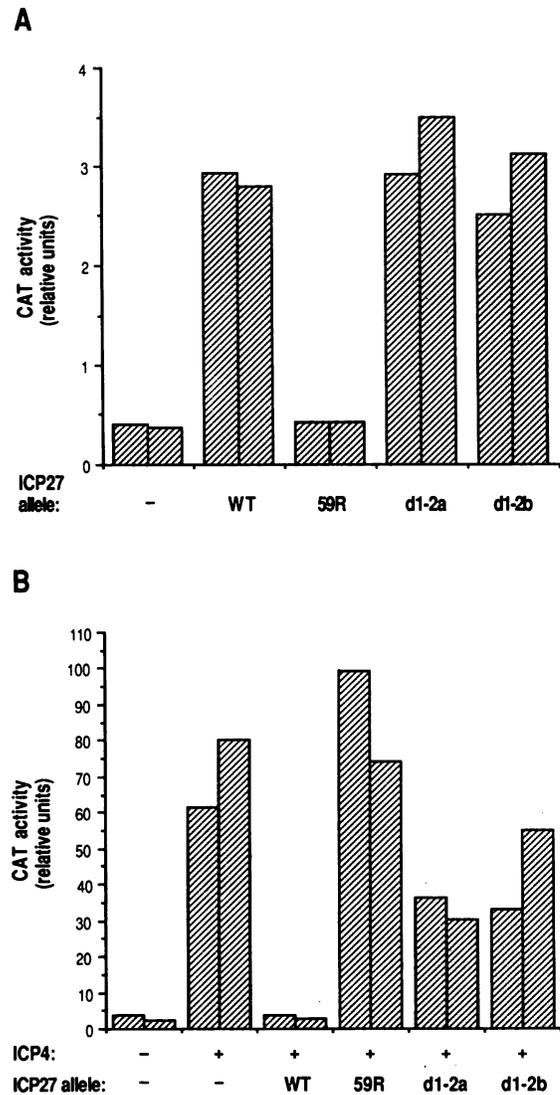


FIG. 2. Evidence that the *d1-2* plasmid mutants are defective for transrepression but not transactivation. WT or mutant plasmids were tested in cotransfection assays for the transactivation (A) or transrepression (B) functions of ICP27. (A) Transactivation assay. Vero cell cultures were transfected in duplicate with 6 μ g of pgBCAT(-175) plus, where indicated, 4 μ g of WT or mutant ICP27 plasmid. The cells were harvested 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 μ g of p8CAT and, where indicated, 2 μ g of pSVSK4, encoding ICP4. Where indicated, 4 μ g of WT or mutant ICP27 plasmid was also added. Analysis and representation of CAT activities is as in panel A.

sion (44). The p8CAT plasmid was transfected alone, with a plasmid encoding ICP4, or with plasmids encoding ICP4 plus WT or mutant ICP27 plasmids. As expected, cotransfection of the ICP4 plasmid greatly transactivated the ICP8-CAT reporter gene (Fig. 2B). Consistent with previous observations, the addition of a plasmid encoding WT ICP27 dramatically inhibited ICP4-mediated transactivation (18- to 31-fold), while the control plasmid pBH-59R had little if any effect. Compared with the WT construct, both pBSd1-2 isolates were defective in the ability to repress 8-CAT

TABLE 1. Complementation of *d27-1* growth by ICP27-encoding plasmids

Plasmid transfected ^a	Virus yield (PFU/ml) ^b	Complementation index (%) ^c
pUC19	$<1.0 \times 10^1$	
pBS27	1.3×10^5	100
pBSd1-2a	2.5×10^3	1.9
pBSd1-2b	1.0×10^4	7.7

^a Approximately 3×10^6 Vero cells were transfected with the plasmids indicated. One day later, the cells were infected with 2 PFU of *d27-1* per cell. The infected cells were harvested after 24 h.

^b Virus titers in the culture lysates (10 ml) were determined by plaque assay on V27 cells.

^c Expressed as yield relative to that for the transfection with pBS27 DNA.

transactivation, showing only two- to threefold repression. These results suggest that the acidic N-terminal region of ICP27 has an important role in the transrepression function of ICP27.

To determine whether the acidic region of ICP27 is involved in an activity necessary for lytic viral replication, we examined whether the mutant plasmids could complement the growth of a viral mutant, *d27-1* (33), which does not express ICP27 because of a large deletion (Fig. 1). Vero cells were transfected with pUC19, pBS27, pBSd1-2a, or pBSd1-2b DNA. The following day, the cells were infected with *d27-1* and allowed to undergo one infectious cycle. Virus yield was determined by plaque assay on a cell line, V27 (33), which is permissive for *d27-1* growth as the result of the expression of a stably transfected ICP27 gene. No viral growth was detected in the culture transfected with pUC19, whereas transfection with pBS27 increased the yield of *d27-1* by more than 4 orders of magnitude (Table 1). Transfection with the pBSd1-2 plasmids resulted in virus yields that were 2 to 8% of the WT value. These results suggest that the N-terminal acidic region of ICP27 is required for a function needed for the efficient replication of the virus in Vero cells.

However, it is possible that deletion of the acidic region of ICP27 causes the mutant polypeptide to be less stable than the WT protein. If so, we could not conclude that the deletion directly affected a regulatory activity of ICP27. To test the stability of the mutant polypeptides, we transfected Vero cells with the WT or mutant plasmids. The expressed proteins were analyzed by SDS-PAGE and Western blot analysis, using a monoclonal antibody specific for ICP27 (Fig. 3A). The pBSd1-2 plasmids encoded smaller ICP27-related proteins with apparent molecular sizes of 52 kDa, consistent with the predicted size. Furthermore, the mutant proteins accumulated to levels that were comparable to that of the WT protein expressed from the pBS27 plasmid. We also used immunofluorescence microscopy to examine the cellular localization of the mutant proteins in transfected cells. Like the WT protein, the mutant proteins were almost exclusively nuclear (34). Therefore, the defects in transrepression and virus complementation exhibited by the *d1-2* plasmid mutants appear to result directly from an altered regulatory function of ICP27.

The acidic amino-terminal region of ICP27 is required for lytic viral growth. To study the function of the acidic N-terminal portion of ICP27 in the viral infection, we used a marker transfer protocol (33) to introduce the *d1-2* allele into the HSV-1 genome in place of the WT ICP27 gene. Marker transfer and growth of the mutant virus stocks were carried

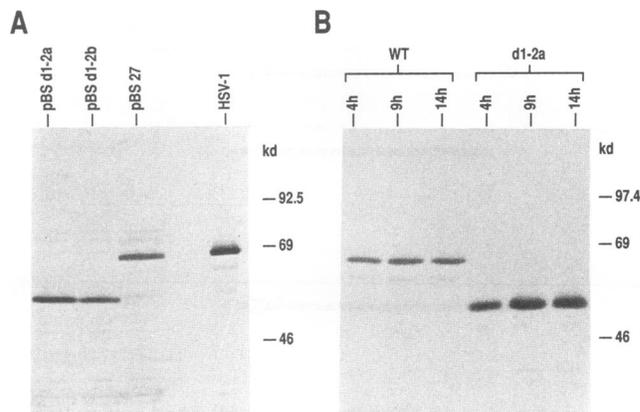


FIG. 3. Western blot analysis of ICP27 polypeptides expressed in transfected and infected cells. (A) Expression in transfected cells. Vero cells were transfected with the plasmids indicated, and the total cell lysates were prepared after 2 days. The proteins in the lysates were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with H1113, a monoclonal antibody specific for ICP27 (1). As a control, proteins from HSV-1-infected Vero cells were electrophoresed in the right-hand lane. The molecular sizes of ¹⁴C-labeled standards are indicated at the left. (B) Expression in infected cells. Vero cells were infected with the WT virus or *d1-2a*, and the total proteins were isolated at the times indicated at the top. Western blot analysis was carried out as for panel A. The sizes of protein standards are indicated at the right.

out in ICP27-expressing V27 cells, so that potential deficiencies in viral growth would be complemented by the expression of WT ICP27. Two independent isolates of the desired mutant were obtained and designated *d1-2a* and *d1-2b*. These mutants were generated using the *d1-2a* and *d1-2b* plasmid DNAs, respectively, as gene donors. Southern blotting experiments confirmed that both viral isolates contained the *d1-2* mutant alleles at the ICP27 locus (34).

The *d1-2* stocks generally failed to form plaques when titrated on Vero cells, indicating that the deletion had inactivated an essential function of ICP27. However, in some experiments the mutant stocks formed very minute plaques on Vero cells. Therefore, to study the effect of the *d1-2* mutation on viral growth in more detail, single-cycle virus growth experiments were performed at a multiplicity of infection of 10 (Table 2). The *d1-2a* and *d1-2b* stocks were both defective for growth in Vero cells in this assay, replicating approximately 100-fold less efficiently than the parental WT strain, KOS1.1. Additional experiments showed that the *d1-2* mutants gave a similar low yield if the infections

TABLE 2. Growth properties of HSV-1 ICP27 mutants

Virus	Virus yield (PFU/cell) ^a	
	Vero cells	V27 cells
KOS1.1	140	170
<i>d1-2a</i>	1.7	33
<i>d1-2b</i>	1.3	90
<i>d27-1</i>	<0.00005	130
n504R	0.021	56

^a Vero cells (2×10^6) were infected at a multiplicity of infection of 10 PFU per cell. The same inocula were used to infect parallel cultures of 3×10^6 V27 cells. The cultures were harvested at 24 hpi, and virus yield was determined by plaque assay on V27 cells.

were incubated for 2 days instead of 1 (34), indicating that the defect in *d1-2* growth was not simply due to a delay in the kinetics of viral growth. Both isolates grew efficiently in V27 cells, demonstrating that the mutation could be complemented in *trans* by the WT ICP27. Although the *d1-2* isolates were defective for growth in Vero cells, they were not nearly as impaired as the ICP27 null mutant *d27-1* and the ICP27 C-terminal mutant *n504R* (Fig. 1), which were reduced in yield in Vero cells by more than 6 and 3 log units, respectively, compared with the WT virus.

To test the possibility that *d1-2* polypeptide was unstable in infected cells, we analyzed the steady-state level of ICP27 proteins produced in the WT and *d1-2a* mutant infections. Total proteins from infected cells were isolated at 4, 9, and 14 hpi and subjected to Western blot analysis (Fig. 3B). The mutant ICP27 protein synthesized in *d1-2a*-infected cells had an apparent molecular size of 51 kDa, similar to the size observed in transfected cells. At all time points tested, the mutant protein accumulated to levels that were equal or greater than the WT protein level. Similar results were observed for the mutant polypeptide expressed by the *d1-2b* virus (34). Furthermore, immunofluorescence experiments indicated that the mutant ICP27 proteins expressed in *d1-2a*- and *d1-2b*-infected Vero cells accumulated predominately in the cell nucleus (34). Therefore, the growth defect of the *d1-2* mutants in Vero cells does not appear to be due to either instability of the mutant protein or a defect in its nuclear localization.

The *d1-2* mutants are deficient in viral DNA replication. The *d1-2* isolates are the first ICP27 virus mutants to be isolated which encode proteins with alterations specifically in the N-terminal half of ICP27. It was therefore of interest to characterize their phenotypes in detail. First, we studied the ability of the *d1-2* isolates to replicate viral DNA. Vero cells were mock infected or were infected with either WT HSV-1, *d1-2a*, or *d1-2b*, the ICP27 null mutant *d27-1*, or the ICP27 C-terminal mutant *n504R*. As a control, the same inocula were used to infect parallel cultures of V27 cells. The cultures were labeled with [³H]thymidine from 9 to 12 hpi. After labeling, the total DNA from the cultures was isolated and equal amounts were analyzed by restriction enzyme digestion (*Eco*RI plus *Xba*I), gel electrophoresis, and fluorography. The results from the Vero cell infections indicated the ICP27 mutants differed significantly in their abilities to replicate viral DNA (Fig. 4A). Both isolates of the *d1-2* mutant showed defects in viral DNA synthesis. Consistent with our previous results (33), the ICP27 null mutant *d27-1* also exhibited a deficiency in viral DNA replication. Densitometric analysis of autoradiograms indicated that the *d1-2a* and *d1-2b* mutants replicated 3- and 8-fold less DNA, respectively, during the labeling period than did the WT virus, while the *d27-1* mutant replicated 12-fold less DNA. The *n504R* mutant, on the other hand, replicated viral DNA as well as did the WT virus. This result is consistent with our previous phenotypic analysis of this mutant (33). All of the viruses replicated viral DNA to similar extents in V27 cells (Fig. 4B), demonstrating that the differences seen in Vero cells were not due to variations in the titers of the inocula. These results indicate that the acidic region of ICP27 is required for normal levels of viral DNA synthesis in infected cells.

The *d1-2* mutants exhibit modest defects in viral gene expression. We next studied viral gene expression in *d1-2*-infected cells. First, we examined the rates of viral protein synthesis at various times after infection. Vero cells either were mock infected or were infected with the WT virus or

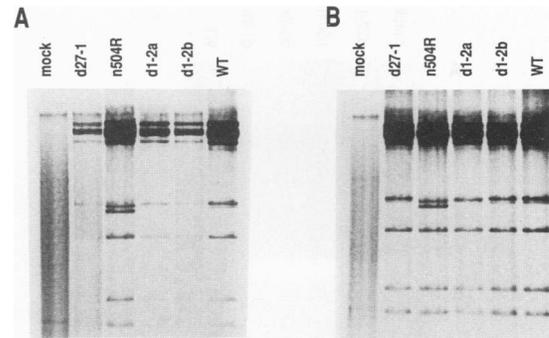


FIG. 4. Viral DNA replication in ICP27 mutant-infected cells. Vero (A) or V27 (B) cells were mock infected or were infected with the viruses indicated at the top. The cells were labeled with [³H]thymidine from 9 to 12 hpi. Total DNA was then purified, and equal amounts were digested with *Eco*RI plus *Xba*I and electrophoresed on a 0.9% agarose gel. The gel was processed for fluorography, dried, and exposed to X-ray film. The *n504R* genome contains an *Xba*I site at the 3' end of the ICP27 gene (Fig. 1) and therefore gives rise to a novel *Eco*RI-*Xba*I restriction fragment.

with ICP27 mutants. At 4, 9, or 14 hpi, the cultures were pulse-labeled with [³⁵S]methionine-cysteine, and the proteins were analyzed by SDS-PAGE and autoradiography (Fig. 5). Both isolates of *d1-2* showed a pattern of viral protein synthesis that differed modestly from the WT pattern

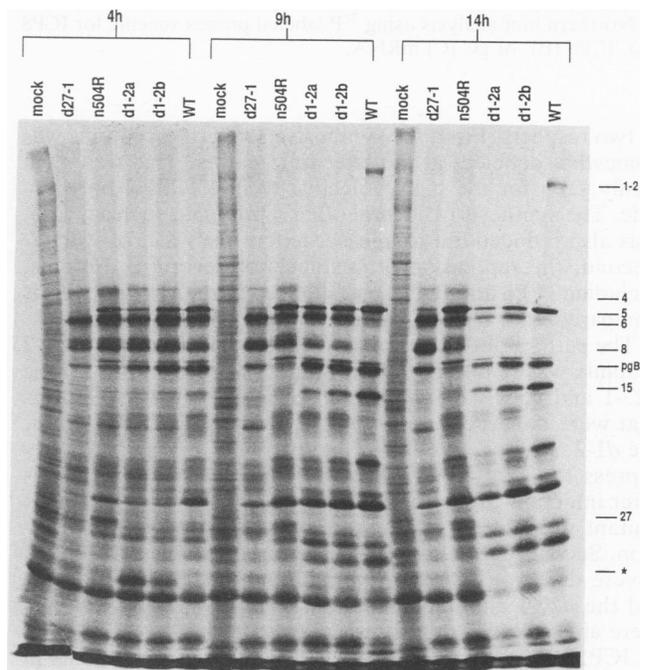


FIG. 5. Protein synthesis in ICP27 mutant-infected cells. Vero cells were mock infected or were infected with the WT virus or ICP27 mutants. At 4, 9, and 14 hpi, the cells were pulse-labeled for 30 min with [³⁵S]methionine-cysteine and then harvested. Equal fractions of each cell lysate were analyzed by SDS-PAGE and autoradiography. Shown at the right are the positions of migration of several HSV-1 proteins. The WT ICP27 polypeptide and the *n504R* mutant ICP27 polypeptide comigrate on this gel, while the *d1-2* mutant ICP27 polypeptides are found at a lower position (indicated by an asterisk).

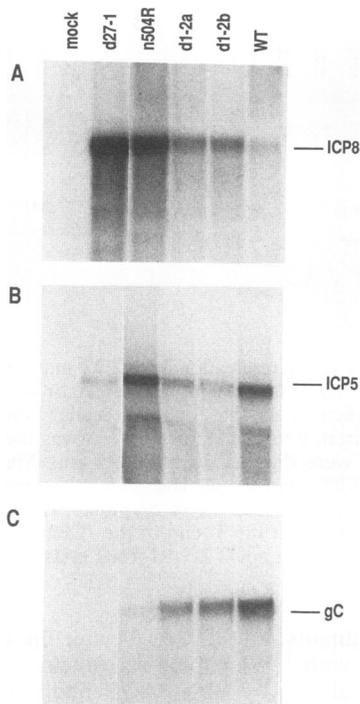


FIG. 6. Accumulation of viral mRNAs in ICP27 mutant-infected cells. Vero cells were mock infected or were infected with the viruses indicated at the top. At 12 hpi, cytoplasmic RNA was prepared. Equal amounts of each RNA preparation were subjected to Northern blot analysis using ^{32}P -labeled probes specific for ICP8 (A), ICP5 (B), or gC (C) mRNA.

in two respects. First, the synthesis of several γ proteins was somewhat deficient at the later time points. This was most easily seen for the high-molecular-weight ICP1-2 polypeptide. The synthesis of several other γ proteins, such as ICP5, was also reduced but to a lesser extent than that of ICP1-2. Second, the repression of synthesis of several β proteins, including ICP6 and ICP8, was delayed in the *d1-2* infections compared with the WT infection.

The pattern of viral protein synthesis exhibited by the *d1-2* mutants was quite different from the patterns shown by the *d27-1* and *n504R* mutants, both of which showed patterns that were consistent with our previous analysis (33). First, the *d1-2* mutants, like the WT virus, were able to efficiently repress the expression of the α protein ICP4 at late times after infection. In contrast, neither the *d27-1* nor the *n504R* mutant was able to efficiently down-regulate ICP4 expression. Second, the *d1-2* mutants appeared to have a much less severe defect in the repression of β protein synthesis than did the *d27-1* and *n504R* mutants. Third, the *d1-2* mutants were able to induce the synthesis of certain γ proteins such as ICP15, which were not induced to appreciable extents in the *d27-1* and *n504R* infections.

To study *d1-2a* and *d1-2b* gene expression in more detail, we examined the accumulation of specific mRNAs from well-characterized β , γ -1, and γ -2 genes. Vero cells were mock infected or were infected with the WT virus or ICP27 mutants. Cytoplasmic RNA was isolated at 12 hpi and analyzed by Northern blot analysis. We first studied the expression of a β mRNA, that encoding the major DNA-binding protein, ICP8 (Fig. 6A). The *d1-2* infections both showed a modest overexpression of ICP8 mRNA compared

with the WT infection. This result was consistent with the protein synthesis analysis, which showed that β protein repression was somewhat delayed in the *d1-2* infections (Fig. 5, 9 hpi). The *d27-1* and *n504R* infections, however, showed a more dramatic overexpression of ICP8 mRNA at 12 hpi, consistent with the high levels of protein synthesis that were observed at late times. The expression of a γ -1 mRNA, that encoding the major capsid protein ICP5, was next examined (Fig. 6B). Expression of ICP5 mRNA was reduced in the *d1-2* infections compared with the WT infection. A similar reduction was seen in the *d27-1* infection. The *n504R* infection, on the other hand, showed WT levels of ICP5 mRNA. The levels of ICP5 mRNA in the various infections correlated qualitatively with the levels of ICP5 protein synthesis that were observed at late times (Fig. 5, 14 h). Finally, we examined the expression of a well-characterized γ -2 mRNA, that encoding the glycoprotein C (gC; Fig. 6C). Accumulation of gC mRNA was only modestly reduced in the *d1-2a* and *d1-2b* infections compared with the WT infection, showing three- and twofold reduction, respectively, as determined by densitometric analysis. In contrast, accumulation of gC mRNA was quite deficient in the *d27-1* and *n504R* infections. In the case of the *d27-1* infection, gC mRNA was undetectable, while the levels in the *n504R* infection were reduced 13-fold compared with the WT infection.

The results presented above demonstrate that the two *d1-2* isolates exhibit similar defects in viral gene expression. Specifically, the *d1-2* mutants show both a reduction in the expression of γ mRNAs and proteins and a delay in the down-regulation of β mRNAs and proteins. These modest deficiencies are in contrast to the more striking defects in gene expression which are exhibited by the ICP27 null mutant *d27-1* and the ICP27 C-terminal mutant *n504R*.

DISCUSSION

A striking feature of the primary sequence of ICP27 is the high proportion of acidic amino acid residues in its N-terminal region. Nearly 40% of the 64 N-terminal residues of ICP27 consist of aspartic or glutamic acid. The presence of an acidic region in this viral gene-regulatory protein is of interest for two reasons. First, acidic regions are found in many viral and cellular transcription factors and have been implicated in transcriptional activation. They are thought to function via an interaction with one or more components of the RNA polymerase II transcription complex (43). Second, several other herpesviruses have been shown to possess open reading frames (ORFs) with some degree of amino acid homology to ICP27. These ORFs include varicella-zoster virus ORF 4 (6), equine herpesvirus 1 ORF 5 (45, 51), Epstein-Barr virus BMLF1 (6), and the herpesvirus saimiri 52-kDa ORF (28). Although the homology between ICP27 and these ORFs is mostly limited to their C-terminal halves, all contain a cluster of acidic residues at their N termini. This observation raises the possibility that the acidic region of ICP27 is involved in a function common to diverse herpesviruses.

The acidic N-terminal region of ICP27 is required for efficient transrepression in transfected cells. To study the function of the acidic region of ICP27, we constructed an ICP27 gene in which the sequences encoding it (codons 12 through 63) were deleted. The mutant gene was tested in a number of transfection assays designed to measure the properties and functions of ICP27 in uninfected cells. These experiments indicated that the N-terminal deletion did not affect the stability or nuclear localization of ICP27 or its

ability to transactivate the expression of a reporter gene. However, the deletion did significantly reduce the ability of ICP27 to transrepress gene expression in transfected cells. This result is noteworthy because previous studies have concluded that the transrepressing activity of ICP27 maps to its C-terminal half (17, 26). Although it is clear that regions in the C-terminal half of the molecule are required for transrepression, the only published data which might argue against an important contribution of the N-terminal half are the experiments of Hardwicke et al. (17). These investigators constructed plasmids having in-frame oligonucleotide insertions at many sites in the ICP27 coding region. It was found that insertions in the N-terminal half of the ICP27 gene, including insertions between residues 28 and 29 and residues 58 and 59, had no effect on transrepression. The combined results thus suggest that the specific amino acid sequence of the N-terminal region of ICP27 may not be as important as a general biochemical feature of this region, such as its high negative charge. If so, the N-terminal region of ICP27 may be similar to the negatively charged domains of transcription factors, many of which do not appear to depend on specific amino acid sequences (23). It will be of interest to determine whether other acidic sequences can substitute for the acidic region of ICP27.

The acidic N-terminal region of ICP27 is required for an essential lytic function. Our plasmid complementation assay suggested that the acidic region of ICP27 is required for efficient viral growth. To test this possibility directly, we introduced the mutant ICP27 allele into the HSV-1 genome in place of the WT gene. Two independently derived isolates of the recombinant virus, *d1-2a* and *d1-2b*, were recovered and separately characterized. This was done to exclude the possibility that an unintended, secondary mutation might contribute to the observed phenotype of the *d1-2* mutant. However, the two isolates exhibited very similar, if not identical, phenotypic properties. In addition, the growth defects of both mutants could be complemented by the WT ICP27 expressed from V27 cells. These results together show that the phenotype displayed by the *d1-2* isolates resulted directly from the engineered mutation.

Single-cycle growth experiments indicated that the virus yield from *d1-2*-infected Vero cells was reduced approximately 100-fold compared with the WT infection. Therefore, we conclude that the acidic N-terminal region of ICP27 is required for an essential lytic function. To gain insight into that function, we examined viral DNA replication and gene expression in *d1-2*-infected cells. We found that the mutant infections differed from the WT infection in two respects. First, the *d1-2* isolates were deficient in viral DNA replication, showing a three- to eightfold reduction in the level of DNA synthesis. Second, the *d1-2* mutants exhibited modest deficiencies in viral gene expression. Specifically, *d1-2*-infected cells showed both a reduction in the level of γ gene expression and a delay in the repression of β gene expression.

Inhibition of HSV-1 DNA synthesis by a variety of means results in both a reduction in γ gene expression and a potentiation of β gene expression (36). Therefore, the simplest hypothesis that would explain the phenotype of *d1-2* is that the primary effect of the mutation is to reduce the amount of viral DNA replication. The reduced amounts of DNA replication in *d1-2*-infected cells might result indirectly in the observed alterations in viral gene expression. However, at present we cannot exclude the possibility that the *d1-2* mutation has a direct effect on gene expression in infected cells. Indeed, our finding that the *d1-2* plasmid

mutant is defective for transrepression in transfected cells is consistent with this latter possibility.

Stimulation of viral DNA replication by ICP27. Our results demonstrate that ICP27 has a specific role in stimulating viral DNA replication in infected cells. However, ICP27 is not one of the seven HSV-1 replication proteins which are thought to participate directly in the DNA replication process (reviewed in reference 48). Indeed, these seven viral proteins are sufficient in transfected cells to direct replication from an HSV-1 origin of replication contained on a plasmid molecule (ori-plasmid) (50). How then does ICP27 stimulate the level of viral DNA replication in infected cells? One possibility is that ICP27 stimulates the expression of one or more of the replication proteins, which are of the β kinetic class. This explanation appears unlikely, however, because the major β genes that we examined, ICP6 and ICP8, were not underexpressed in either *d1-2* or *d27-1* infections. In fact, these genes were overexpressed at late times. This latter observation raises a second possibility, which is that the overexpression of β proteins inhibits viral DNA replication to some extent. However, this explanation also appears unlikely because the *n504R* mutant shows elevated levels of β gene expression but is not defective for DNA replication. A third possibility, and the one that we favor, is that ICP27 stimulates DNA replication not by an effect on viral gene expression but in a more direct capacity, perhaps by altering the posttranslational modification of host or viral proteins (26, 32, 44). Further experiments will be required to distinguish between these and other possibilities.

It is of interest that ICP27 was identified as a stimulatory factor in the original ori-plasmid replication assays used to map the HSV-1 replication genes (50). At the time, it was suggested that ICP27 stimulated plasmid replication by transactivating one or more of the seven HSV-1 replication genes. However, we have shown that the *d1-2* allele is not defective for transactivation in a transfection assay but does lead to a viral DNA replication defect when introduced into a recombinant virus. This finding suggests that ICP27 may stimulate DNA replication in the ori-plasmid replication assays by a mechanism other than transactivation. It will be of interest to determine whether the *d1-2* protein can stimulate the replication of an ori-plasmid in transfected cells.

ICP27 mediates multiple activities in infected cells. We previously suggested that ICP27 carries out two distinct regulatory functions during the HSV-1 infection (33). This hypothesis was based on the phenotypic analysis of several viral ICP27 mutants, including the null mutant *d27-1* and the C-terminal mutant *n504R*. The *d27-1* mutant showed defects in viral DNA replication, γ -1 gene expression, and γ -2 gene expression, whereas the *n504R* mutant exhibited only defects in γ -2 gene expression. Therefore, we proposed that ICP27 mediates two genetically separable activities in infected cells: (i) an activity that stimulates viral DNA replication and γ -1 gene expression and (ii) an activity that transactivates γ -2 genes, independent of viral DNA replication. The phenotype of the *d1-2* mutant can be considered in the context of this model if we hypothesize that it is defective in the first but not the second activity. This mutant exhibits a deficiency in γ -1 gene expression and viral DNA replication and shows a modest reduction in γ -2 gene expression. However, the reduction in γ -2 gene expression in *d1-2*-infected cells is consistent with the three- to eightfold defect in viral DNA replication that is observed and contrasts quite dramatically with the more severe reductions in γ -2 gene expression which are observed in *d27-1*-infected

cells (in which DNA replication is defective) and *n504R*-infected cells (in which DNA replication is normal).

However, some of our results are not entirely consistent with these interpretation. For example, *d27-1*-infected cells show a striking defect in the induction of ICP5 and at least two other proteins at early times (Fig. 5, 4 hpi). This effect is not observed in cells infected by *d1-2* or *n504R*. Therefore, *d27-1*- and *d1-2*-infected cells induce the γ -1 protein ICP5 and some other proteins with quite different kinetics, even though they appear to be similarly defective for viral DNA replication. Thus, ICP27 may induce γ -1 genes by multiple mechanisms. In addition, if the defective functions of *d1-2* and *n504R* are completely independent, then two mutants should be capable of intragenic complementation. We have failed to observe such complementation (34), suggesting that they are not completely independent.

The current evidence suggests that ICP27 is a multifunctional protein. It appears to regulate viral gene expression at both the transcriptional (18, 25) and posttranscriptional (38, 42) levels and is required for efficient viral DNA replication (25, 33; this study). The functional complexity of ICP27 is underscored by our genetic analyses. Of the six recombinant viruses that we have isolated, five display demonstrably different phenotypes with respect to viral gene expression and DNA replication (33; this study). These results suggest that ICP27, like other viral regulatory proteins such as the adenovirus E1A proteins (10) and SV40 T antigen (9), is composed of multiple functional domains which mediate distinct activities. Although previous studies have suggested that the important regulatory activities of ICP27 are mediated by its C-terminal half, our analysis of the *d1-2* mutant indicates that the N-terminal half also has an essential role in regulation. The definitive mapping of the functional domains of ICP27 will require the isolation and analysis of additional ICP27 gene mutations.

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