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Genetic Evidence for Two Distinct Transactivation Functions of the Herpes Simplex Virus α Protein ICP27

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Infected-cell protein 27 (ICP27) is a herpes simplex virus type 1 α, or immediate-early, protein involved in the regulation of viral gene expression. To better understand the function(s) of ICP27 in infected cells, we have isolated and characterized viral recombinants containing defined alterations in the ICP27 gene. The mutant virus *d*27-1 contains a 1.6-kilobase deletion which removes the ICP27 gene promoter and most of the coding sequences, while *n*59R, *n*263R, *n*406R, and *n*504R are mutants containing nonsense mutations which encode ICP27 molecules truncated at their carboxyl termini. All five mutants were defective for lytic replication in Vero cells. Analysis of the mutant phenotypes suggests that ICP27 has the following regulatory effects during the viral infection: (i) stimulation of expression of γ-1 genes, (ii) induction of expression of γ-2 genes, (iii) down regulation of expression of α and β genes late in infection, and (iv) stimulation of viral DNA replication. Cells infected with the mutant *n*504R expressed wild-type levels of γ-1 proteins but appeared to be unable to efficiently express γ-2 mRNAs or proteins. This result suggests that ICP27 mediates two distinct transactivation functions, one which stimulates γ-1 gene expression and a second one required for γ-2 gene induction. Analysis of the mutant *n*406R suggested that a truncated ICP27 polypeptide can interfere with the expression of many viral β genes. Our results demonstrate that ICP27 has a variety of positive and negative effects on the expression of viral genes during infection.

The genes of herpes simplex virus type 1 (HSV-1) are expressed in a highly ordered fashion during lytic infection, and several viral and cellular proteins involved in their regulation have been identified. Although the role of cellular proteins in this process is not well understood, the ability to isolate viral mutants has allowed detailed analyses of the functions of some of the viral regulatory proteins. Here we describe the isolation and characterization of viral mutants defective in an essential viral regulatory factor, infected-cell protein 27 (ICP27).

The 70 to 80 genes of HSV-1 are transcribed by host cell RNA polymerase II and are expressed in four sequential classes: α, β, γ-1, and γ-2 (for a review, see reference 50). Transcription of the five α, or immediate-early, genes does not require viral protein synthesis but is stimulated by a protein component of the virus particle, Vmw65 (also called ICP25, α *trans*-inducing factor, and VP16). The next genes expressed are the β, or early, genes. Most of the β genes encode proteins involved in viral DNA replication, and viral DNA synthesis begins shortly after their expression. Soon after β gene expression, the γ-1 genes are transcribed. The expression of γ-1 genes is enhanced as viral DNA is replicated, but significant γ-1 gene expression can occur prior to, or in the absence of, viral DNA replication. Finally, the γ-2 genes are expressed. The γ-2 genes are not expressed at detectable levels until after viral DNA replication.

The expression of the β and γ classes of genes requires the expression of functional α proteins (22). In particular, expression of the 175-kilodalton (kDa) protein ICP4 is required for transcription of β and γ genes (8, 15, 35, 51). ICP4 is a multifunctional protein with intrinsic DNA-binding activity, but the mechanism by which it activates β and γ genes is not yet understood (24, 32, 34, 46). The 110-kDa α protein ICP0 may also play a regulatory role during lytic infection, because viral mutants bearing deletions in the ICP0 gene grow poorly, especially at low multiplicities of infection (42, 48).

Genetic studies have indicated that a third α protein, the 63-kDa ICP27, is involved in the regulation of expression of HSV-1 genes. HSV-1 mutants with temperature-sensitive (*ts*) lesions in the ICP27 gene exhibit reduced expression of many γ-1 genes and are unable to induce expression of γ-2 genes at the nonpermissive temperature (41). The defects in γ gene expression are not due to defects in viral DNA replication, because the *ts* mutants replicate their DNA nearly as well as the wild-type (WT) virus does. Recently, a replication-defective virus bearing a deletion in the ICP27 gene was isolated and characterized (29). Under nonpermissive conditions this mutant replicated a reduced amount of viral DNA, overexpressed certain β proteins, was defective in inducing many γ-1 proteins, and failed to induce γ-2 proteins.

Transient-expression experiments have also provided evidence that ICP27 modulates HSV-1 gene expression. In such experiments, reporter genes are transfected into uninfected cells together with the ICP27 gene and, in some cases, other HSV-1 regulatory genes. ICP27 can transactivate several HSV-1 promoters, including those for the alkaline exonuclease (β), glycoprotein B (gB) (β or γ-1), ICP5 (γ-1), and glycoprotein C (gC) (γ-2) genes (9, 39, 44, 49). In most cases this effect requires coexpression of ICP4 and/or ICP0; however, under certain conditions ICP27 alone can transactivate the gB promoter (39). ICP27 can also negatively affect the expression of several HSV-1 α, β, and γ promoters in transfection assays (3, 44, 49). In most cases, ICP27 has little or no effect on the basal expression of these promoters but rather acts to block the ability of a cotransfected ICP4 or ICP0 gene to induce expression from the promoters. Host cell factors and other variables are likely to play a role in the response of a particular promoter to ICP27 regulation in

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transfected cells, because various laboratories have reported quite different effects of ICP27 on certain HSV-1 promoters.

We recently constructed four mutant plasmids that encode ICP27 molecules truncated to various extents at their carboxyl termini. The mutant genes were tested in transient-expression assays to determine the extent to which the truncated ICP27 polypeptides were able to modulate viral gene expression (40). These experiments indicated that ICP27 possesses separable positive and negative regulatory activities. Using a similar approach, Hardwicke et al. (17) have also provided evidence that the two regulatory activities of ICP27 can be genetically separated. To see how the same mutant proteins we previously characterized affect gene expression in the context of the infected cell, we have replaced the WT gene in the viral chromosome with each of the mutant genes. In addition, we have isolated a viral mutant that bears a large deletion in the ICP27 gene and makes no detectable ICP27 mRNA or protein. The analysis of these mutants provides further evidence that ICP27 has both positive and negative effects on the expression of HSV-1 genes. Furthermore, our results indicate that ICP27 possesses two genetically separable transactivation functions. One activity stimulates, but is not required for, the expression of the γ-1 genes, while the second activity is required for expression of the γ-2 genes.

MATERIALS AND METHODS

Cells, viruses, infections, and transfections. All infections and transfections were carried out in Vero or V27 cells. Vero cells were obtained from the American Type Culture Collection, Rockville, Md.; the derivation of V27 cells is described below. HSV-1 KOS1.1, originally provided by M. Levine (University of Michigan, Ann Arbor), was the WT strain used in all experiments. Infections were carried out at a multiplicity of 10 PFU per cell. Disodium phosphonoacetate (a gift from Abbott Laboratories, North Chicago, Ill.), when used, was added to the inocula and overlay media at a concentration of 400 μg/ml. Transfection of viral DNA for marker transfer was performed in V27 cells by using the calcium phosphate precipitation method previously described (39).

The V27 cell line, which carries a stably integrated copy of the ICP27 gene, was isolated in the following manner. Subconfluent 100-mm-diameter dishes of Vero cells were transfected with 0.8 μg of pSV2neo (47), a plasmid conferring resistance to the drug G418, and either 4 or 10 μg of pBH27 (39), a plasmid encoding HSV-1 ICP27. Two days later, the cells were passaged 1:9 in medium containing 600 μg of G418 per ml. G418-resistant colonies were isolated 2 to 4 weeks later and grown into mass culture. The cell lines were tested for ICP27 expression on the basis of their ability to support the plaque formation of the ICP27 mutants *ts*Y46 (41) and *ts*LG4 (43) at the nonpermissive temperature. Six of seventeen isolates tested positive in this assay. One of these cell lines, designated V27, was used for the isolation of ICP27 mutants. Southern blot analysis indicated that V27 cells contained approximately one copy of the ICP27 gene per haploid genome equivalent (results not shown).

Isolation of the mutant virus *d27-lacZ1*. The plasmid pPs27pd1 (40) contains a 6.1-kilobase (kb) *Pst*I insert derived from HSV-1 genomic DNA. This fragment contains the entire ICP27 gene, as well as adjoining sequences (Fig. 1A). Derivatives of pPs27pd1 which contained ICP27 gene deletions and *lacZ* gene insertions were constructed in the following manner. pPs27pd1 was linearized in the ICP27 coding region by digestion with *Sal*I. The DNA was then treated with nuclease BAL 31 such that approximately 0.5 kb was digested from each DNA end, and the DNA ends were repaired by a fill-in reaction using the Klenow fragment of *Escherichia coli* DNA polymerase (2). The DNA was ligated to *Bgl*II linker DNA (New England BioLabs, Inc., Beverly, Mass.) and then digested with *Bgl*II, religated, and used to transform *E. coli*. Plasmid DNA from several transformants was pooled, digested with *Bgl*II, and ligated to a 3.1-kb *Bam*HI fragment containing the *lacZ* coding region derived from pMC1871 (45). Following ligation, the DNA was digested with *Bgl*II and used to transform *E. coli*. Four plasmid isolates were obtained which had the *lacZ* gene inserted in the same orientation as the ICP27 gene.

Each of the four plasmid DNAs was digested with *Pst*I,

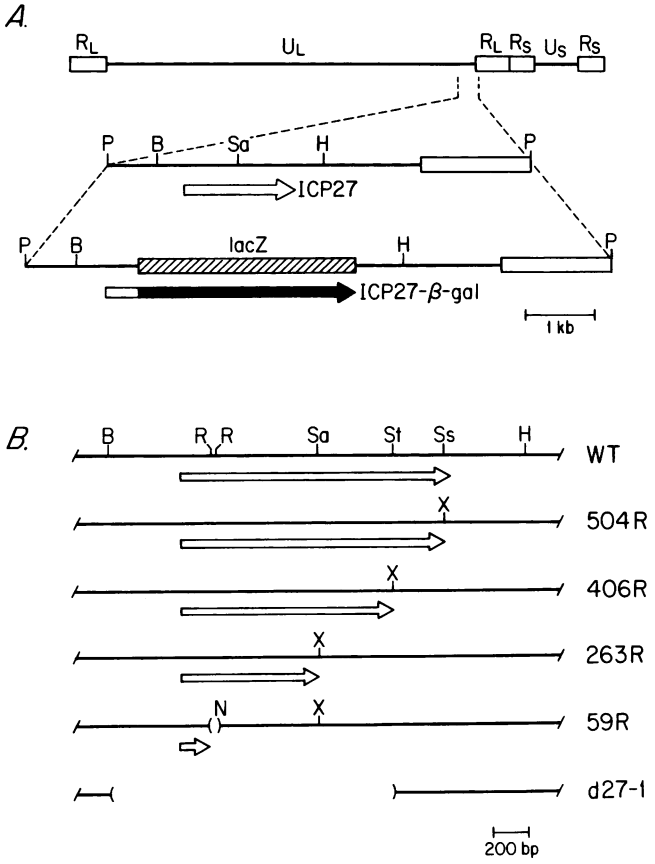

FIG. 1. Location and structure of WT and mutant ICP27 genes. (A) Structures of the WT and the *lacZ* insertion mutant genes. Shown at the top is a representation of the prototype arrangement of the HSV-1 genome. Shown below is a *Pst*I restriction fragment from the WT and the *d*27-*lacZ*1 genome. The narrow lines denote unique (U) regions of the viral genome, the open bars denote repeat regions (R), and the hatched bar denotes *E. coli lacZ* sequences. The upper arrow represents the coding sequences for the 63-kDa ICP27, and the bottom arrow represents the coding sequences for the approximately 137-kDa ICP27-β-galactosidase fusion protein. (B) Structures of the WT, nonsense, and deletion mutant genes. ICP27 mutant genes were constructed by deleting restriction fragments (parentheses) or inserting *Xba*I or *Nhe*I oligonucleotide linkers (X and N, respectively) containing stop codons in all three reading frames. The arrows represent either the WT ICP27 protein (top) or the truncated forms of ICP27 encoded by the nonsense mutants. Restriction sites: P, *Pst*I; B, *Bam*HI; Sa, *Sal*I; H, *Hpa*I; R, *Rsr*II; St, *Stu*I; Ss, *Ssp*I; X, *Xba*I; N, *Nhe*I.

individually mixed with WT HSV-1 DNA, and transfected into V27 cells. After 4 days, the cultures were harvested and the resulting virus stocks were plated onto V27 cells under a liquid overlay of 199 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 1% newborn calf serum and 0.1% human immunoglobulin. After 2 days, the medium was replaced with 199 medium containing 1% newborn calf serum, 0.5% agarose, and 300 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml. One of the four stocks gave rise to a high percentage (approximately 3%) of blue plaques, presumably because the cotransfected plasmid had encoded an in-frame ICP27 gene-*lacZ* gene fusion. A blue plaque arising from this culture was purified three times, and the resulting virus clone was designated *d*27-*lacZ*1. Southern analysis of *d*27-*lacZ*1 DNA indicated that the WT ICP27 gene had been replaced with the ICP27-*lacZ* fusion gene (Fig. 1A). In addition, Southern analysis of viral DNA, as well as restriction analysis of the parental plasmid (designated pPsd27-*lacZ*1), indicated that approximately 0.8 kb had been deleted from the ICP27 gene in *d*27-*lacZ*1.

Isolation of viruses carrying ICP27 deletion and nonsense mutations. Deletion and nonsense mutations in the ICP27 gene were engineered into the pPs27pd1 plasmid (Fig. 1B). The plasmids containing the 406R and 504R mutations, pPs-406R and pPs-504R, respectively, were constructed as described previously (40). The plasmid pPsd27-1 was constructed by digesting pPs27pd1 with *Bam*HI and *Stu*I, filling in the 3′ recessed *Bam*HI DNA ends by using the Klenow fragment of *E. coli* DNA polymerase, and recircularizing the large DNA fragment with DNA ligase. The plasmids pPs-59R and pPs-263R were constructed by substituting the mutant 2.4-kb *Bam*HI-*Sst*I fragments from the plasmids pBH-59R and pBH-263R (40) for the WT 2.4-kb *Bam*HI-*Sst*I fragment of pPs27pd1.

Recombinant viruses were constructed by using a marker transfer strategy. The plasmid DNAs described above were digested with *Pst*I, individually mixed with *d*27-*lacZ*1 viral DNA, and transfected into V27 cells. The progeny viruses were harvested after 3 to 5 days and plated onto V27 cells in the presence of X-gal, as described above. Clear plaques, which were observed at frequencies of 0.5 to 5%, were picked and screened to determine whether they had acquired the ICP27 gene mutations.

Plaque isolates were screened in two ways. Plaque isolates *d*27-1, *n*59R, and *n*263R were purified three times in V27 cells and then used to infect V27 cells. Crude viral DNA was prepared from the infected cells by the method of Gao and Knipe (11). The viral DNAs were digested with *Xba*I and *Bam*HI and examined by agarose gel electrophoresis for the pattern of DNA fragments diagnostic of a particular recombinant. For *n*406R and *n*504R, initial plaque isolates were used to prepare small virus stocks. These stocks were used to infect Vero cells grown on glass cover slips. The infected cells were fixed and stained for immunofluorescence by using an anti-ICP27 monoclonal antibody, H1113 (1). Because the *d*27-*lacZ*1 ICP27-β-galactosidase fusion protein does not react with this antibody, a positive signal was indicative of the expected recombinant. Positive isolates of *n*406R and *n*504R were then plaque purified two more times. Large stocks of all virus mutants were prepared in V27 cells.

Characterization of viral mutants. Viral DNAs for Southern blotting were prepared as described above. Southern blotting and hybridizations were carried out by standard procedures (27). The probe used was the plasmid pPs27pd1, which was labeled with [α-^{32}P]dCTP by the random primer technique using the Klenow fragment of *E. coli* DNA polymerase (2) and random oligonucleotides (Boehringer Mannheim).

To analyze viral DNA replication, Vero cells were mock-infected or infected with various mutants. After a 1-h adsorption period, the monolayers were washed extensively with warm medium to remove unadsorbed virus. Total DNA was prepared by the method of Challberg (5) either immediately after adsorption and washing or at 16 h postinfection (p.i.). The purified DNA was quantitated by UV absorption at 260 nm. Fivefold serial dilutions of the DNA were made and denatured in 100 mM sodium hydroxide for 30 min at room temperature. An equal volume of 12× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) was added, and the DNA was aspirated onto nitrocellulose by using a slot-blot manifold (Schleicher & Schuell, Keene, N.H.). The baked filters were hybridized to ^{32}P-labeled HSV-1-specific probes prepared by random primer labeling. The probe was either pSHZ (33), containing the ICP0 gene, or pRB3441 (31), containing the gene for Vmw65. To quantitate the data, the slots were cut out and the radioactivity was measured by liquid scintillation counting. For a given filter, the number of counts per minute bound to slots of mock-infected-cell DNA was averaged and the mean value was subtracted from the values obtained for the infected-cell DNA slots. For the final quantitation of the data, a slot from each dilution series was chosen that showed hybridization in the linear range of the assay (in practice, this was between 500 and 2,000 cpm hybridized). The number of counts per minute bound to this slot was multiplied by the dilution factor to derive a number, in counts per minute, which was used to quantitate the amount of HSV-1 DNA in each sample.

Western blot (immunoblot) and immunofluorescence analyses using the monoclonal antibody H1113 were carried out by methods described previously (36, 40). For analysis of viral protein synthesis, infected cells were labeled with 15 μCi of [^{35}S]methionine per ml for 30 min. Harvesting of infected-cell proteins and sodium dodecyl sulfate (SDS)-gel electrophoresis in 9.25% polyacrylamide gels were performed by methods previously described (13, 26).

Cytoplasmic RNAs for Northern (RNA) blot analysis were isolated by extraction in phenol-chloroform of the 0.5% Nonidet P-40 supernatant fractions, followed by ethanol precipitation (25). The RNA was then suspended, digested with RNase-free DNase I (Bethesda Research Laboratories, Gaithersburg, Md.), phenol-chloroform extracted, and ethanol precipitated. Ten micrograms of each RNA sample was subjected to electrophoresis through denaturing formaldehyde-agarose gels (27). Following electrophoresis, the RNA was transferred to GeneScreen filters (DuPont, NEN Research Products, Boston, Mass.). Hybridization of ^{32}P-labeled probes, radiolabeled by the random primer method, was performed by methods described previously (38). The plasmids used for probes were pBH27 (ICP27 gene probe [39]), pK1-2 (ICP4 gene probe [7]), and pEcoRI-BamHI-I-I (gC gene probe [10]). Autoradiograms were analyzed densitometrically with an Ultrascan laser densitometer and online integrator (LKB Instruments, Inc., Rockville, Md.).

RESULTS

Construction of HSV-1 ICP27 mutants. Our goal was to construct and characterize HSV-1 mutants containing defined mutations in the ICP27 gene. It was expected, how-

ever, that many HSV-1 ICP27 mutants would have a lethal phenotype. To propagate such mutants, we first isolated a cell line derived from Vero cells, V27, which contained an integrated copy of the ICP27 gene (see Materials and Methods for details). Because the V27 cell line efficiently complemented the growth of HSV-1 ICP27 *ts* mutants at the nonpermissive temperature (data not shown), we expected that it would serve as an efficient host for the isolation of ICP27 mutants.

Insertion of the *E. coli lacZ* gene into the HSV-1 chromosome has been a useful tool in the isolation of viral mutants (4, 16). Viral plaques expressing β-galactosidase, the product of the *lacZ* gene, can be identified on the basis of their color (blue) in the presence of X-gal, a chromogenic substrate for β-galactosidase. Our strategy was first to isolate an HSV-1 mutant expressing β-galactosidase because of an in-frame insertion of the *lacZ* gene into the ICP27 coding sequences. This virus could then serve as a recipient in marker transfer experiments to introduce specifically mutated ICP27 alleles into the viral genome. Recombinants containing the newly introduced ICP27 genes could be identified as clear plaques against a background of parental blue plaques.

To construct the HSV-1 *lacZ* insertion mutant, a recombinant plasmid was constructed in which the *lacZ* coding region was inserted into a deleted version of the ICP27 gene. This fusion gene was then cotransfected into V27 cells with infectious HSV-1 DNA. When the progeny viruses from this transfected culture were plated onto V27 cells in the presence of X-gal, approximately 3% of the plaques were blue. A blue plaque was picked, and the resulting virus clone was designated *d*27-*lacZ*1. Southern blot analysis indicated that *d*27-*lacZ*1 has a genomic structure consistent with the replacement of the WT ICP27 gene with the ICP27-*lacZ* fusion gene (Fig. 1A; data not shown). In addition, *d*27-*lacZ*1-infected cells did not express the WT ICP27 but instead expressed a polypeptide of approximately 137 kDa, consistent with the size predicted for the ICP27-β-galactosidase fusion protein (data not shown). The stock of *d*27-*lacZ*1 virus was unable to form plaques on Vero cells ($<2 \times 10^3$ PFU/ml) but formed plaques efficiently on V27 cells (2×10^8 PFU/ml).

Construction of HSV-1 mutants containing deletion or nonsense mutations in the ICP27 gene. Five plasmids were constructed which contained deletions or nonsense codon insertions in the ICP27 gene (Fig. 1B). These plasmid DNAs were cleaved to release the viral DNA inserts and cotransfected with *d*27-*lacZ*1 DNA into V27 cells. The viral progeny from this marker transfer experiment were plated onto V27 cells in the presence of X-gal, and a fraction (approximately 1 to 5%) formed clear plaques. Viruses which formed clear plaques were isolated and screened for the presence of the newly introduced ICP27 alleles by an immunofluorescence assay or by DNA restriction analysis (see Materials and Methods for details). For each mutant, a positive plaque was purified three times and grown into a large stock in V27 cells. The potential ICP27 deletion mutant was designated *d*27-1. The potential nonsense codon insertion mutants were designated *n*59R, *n*263R, *n*406R, and *n*504R; the numbers in these designations correspond to the number of amino-terminal ICP27 residues expected to be present in each truncated protein. For comparison, the WT protein consists of 512 amino acid residues.

The recombinant viral genomes were characterized by Southern blot analysis to confirm that the intended mutations had been introduced into the viral genome. Viral DNA isolated from infected V27 cells was digested with *Pst*I and *Xba*I, separated by agarose electrophoresis, and transferred to a nylon filter. The filter was hybridized with the 6.1-kb *Pst*I HSV-1 DNA fragment containing the WT ICP27 gene. Because this fragment includes some L-component-repetitive sequences (Fig. 1A), WT HSV-1 DNA contained two hybridizing fragments, the 6.1-kb ICP27 fragment and a 3.3-kb fragment presumably derived from the other U_L-R_L junction (Fig. 2). In contrast, all five ICP27 mutant DNAs lacked the 6.1-kb fragment but contained the 3.3-kb fragment. The *d*27-1 DNA contained a new fragment of approximately 4.6 kb, consistent with its expected 1.6-kb deletion. The four remaining mutant DNAs all contained two new bands, the sizes of which added up to approximately 6.1 kb, consistent with the insertion of an *Xba*I site at the expected position in each mutant genome. Furthermore, none of the mutant genomes contained the 8.4-kb *Pst*I fragment, which would be expected for the *d*27-*lacZ*1 parental DNA (Fig. 1A).

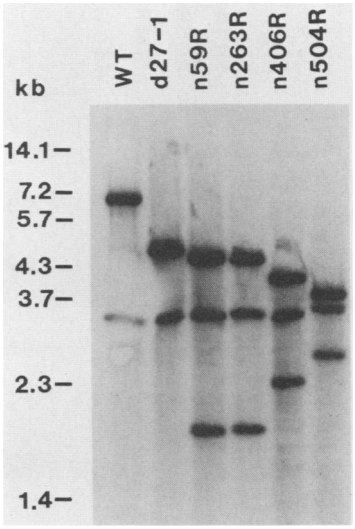

FIG. 2. Southern blot analysis of ICP27 mutant genomes. Purified viral DNAs were digested with *Pst*I and *Xba*I, subjected to electrophoresis in an agarose gel, and transferred to a nylon filter. The filter was probed with ^{32}P-labeled DNA from a plasmid which contains a 6.1-kb *Pst*I insert from the region of the HSV-1 genome encoding ICP27 (Fig. 1A). Shown at the left are the positions of DNA size standards.

Plaque assays were performed to determine whether the mutants were able to grow in Vero cells, a cell line used routinely for growth of HSV-1. All five mutants were unable to form plaques on Vero cells at the lowest dilution which

TABLE 1. Growth of HSV-1 ICP27 mutants[a]

Virus	Viral titer (PFU/ml)	
	Vero cells	V27 cells
KOS1.1 (WT)	7×10^8	5×10^8
*d*27-1	$<2 \times 10^3$	3×10^8
*n*59R	$<2 \times 10^3$	3×10^8
*n*263R	$<2 \times 10^3$	1×10^8
*n*406R	$<2 \times 10^3$	2×10^8
*n*504R	$<2 \times 10^3$	3×10^8

[a] Viral stocks were titrated by plaque assay on the cell lines indicated.

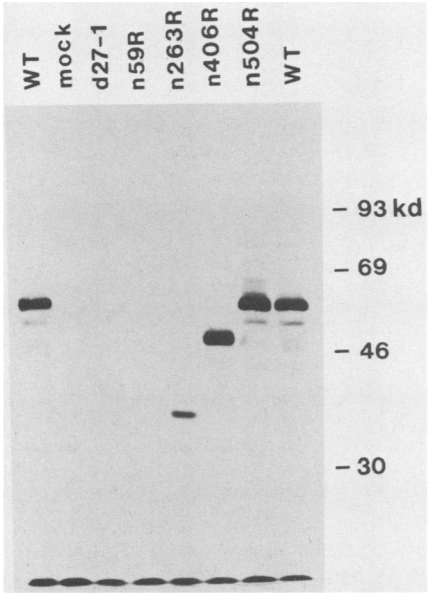

FIG. 3. Western blot analysis of ICP27-related polypeptides expressed in mutant virus-infected cells. Total proteins were prepared from infected Vero cells at 10 h p.i., separated by SDS-PAGE, and electrophoretically transferred to a nitrocellulose filter. The filter was probed with H1113, a monoclonal antibody specific for ICP27. Shown at the right are the positions of ^{14}C-labeled molecular size markers. kd, Kilodaltons.

could be tested (Table 1) (lower dilutions destroyed the cell monolayer). All of the mutants, however, efficiently formed plaques on V27 cells. Because the only known intact HSV-1 gene resident in the V27 genome is the ICP27 gene, these results indicate that the lethal defect of each mutant is complemented in *trans* by expression of the WT ICP27 expressed from V27 cells. Therefore, the observed defect in the ability of each mutant to replicate appears to be the result of the ICP27 mutation that was introduced, not the result of any secondary mutations mapping outside of the ICP27 gene.

Expression and localization of the mutant ICP27 polypeptides. The viral mutants were next analyzed for expression of ICP27-related polypeptides. Vero cells were either mock-infected or infected with virus, and cell extracts were prepared at 10 h p.i. The proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and probed with H1113, a monoclonal antibody directed against ICP27 (1) (Fig. 3). No ICP27-related polypeptides were detected in extracts of mock-, *d*27-1-, or *n*59R-infected cells. An approximately 38-kDa protein was detected in the extracts of *n*263-infected cells, and an approximately 52-kDa protein was present in the extracts of *n*406R-infected cells. Cells infected with *n*504R produced an ICP27-related polypeptide which comigrated with the 63-kDa WT protein. The sizes of the truncated proteins were in rough agreement with the predicted sizes based on the DNA sequence of the ICP27 gene (30).

We previously used immunofluorescence microscopy to examine the intracellular localization of the 263R, 406R, and 504R polypeptides when they were expressed from plasmids in transfected cells (40). All three mutant polypeptides localized to the cell nucleus but differed in their patterns of intranuclear accumulation. Thus, it was of interest to determine the intracellular distribution of the mutant proteins when they were expressed in infected cells. At 4 h p.i., infected Vero cells were fixed and processed for immunofluorescence microscopy using the monoclonal antibody H1113. Cells infected with the WT virus showed nuclear staining (Fig. 4C and D). In most cells, ICP27 localized throughout the cell nucleus but often showed one or a few areas of more intense staining. These areas did not correspond to any structures, including nucleoli, which were visible by phase-contrast microscopy. No immunofluorescence staining above background levels was detected in cells infected with *d*27-1 or *n*59R (data not shown). Cells infected with *n*263R exhibited nuclear staining and, like WT virus-infected cells, showed certain areas of more intense staining (Fig. 4E and F). However, in this case these areas appeared by phase-contrast microscopy to correspond to nucleoli. Cells infected with *n*406R also showed nuclear staining, but the pattern of staining differed from that of WT virus-infected cells in two respects (Fig. 4G and H). First, in most cells the *n*406R protein appeared to be largely excluded from the nucleolar regions. Second, many *n*406R-infected cells showed a rather punctate pattern of staining, with the *n*406R protein being concentrated in globular clusters in the nucleus. Cells infected with *n*504R also showed nuclear staining, but in this case the staining was throughout the nucleus and much more diffuse than in WT virus-infected cells. These results indicate that the *n*263R, *n*406R, and *n*504R proteins efficiently localize to the cell nucleus in infected cells but differ in their patterns of intranuclear accumulation. Furthermore, the intranuclear staining patterns of the mutant proteins in infected cells are similar to the patterns seen in transfected cells (40).

Viral DNA replication by the ICP27 mutants. We next determined the phenotypes of the ICP27 gene mutants with regard to viral DNA replication. An HSV-1 mutant, *d*102, which contains a large deletion in the ICP8 gene and therefore is unable to replicate its DNA (11), was included in these experiments as a negative control for viral DNA replication. Total DNA was isolated from mock- or HSV-1-infected Vero cells immediately after virus adsorption (1 h p.i.) or late in infection (16 h p.i.). Fivefold serial dilutions of the DNA were bound to a nitrocellulose filter, which was then hybridized with a radiolabeled HSV-1-specific DNA probe (Fig. 5). The signal from the input viral DNA (1 h p.i.) was readily detected by this method. As expected, WT virus-infected cells showed a substantial amplification of viral DNA during the course of infection. In contrast, the signal from *d*102-infected cells decreased slightly during infection, consistent with the known requirement for ICP8 in viral DNA replication. Compared with the DNA-negative phenotype of *d*102, it was apparent from this experiment that all five ICP27 gene mutants were able to replicate viral DNA to an appreciable extent during the course of infection.

To quantitate these results, the amount of radioactivity hybridizing to each slot was measured by scintillation counting, and the relative amounts of HSV-1 DNA in each sample were determined. The results of this and a repeat experiment, in which a different HSV-1-specific probe was used, are summarized in Table 2. The results of both experiments were similar and indicated that the ICP27 gene mutants fell into two phenotypic classes with respect to DNA replication. The first class exhibited a partial defect in viral DNA amplification (6 to 38% of the WT level) and consisted of the mutants *d*27-1, *n*59R, *n*263R, and *n*406R. The second class, consisting of the *n*504R mutant, exhibited a WT phenotype for viral DNA replication. It is important to note that these experiments measured the level of viral DNA accumulation

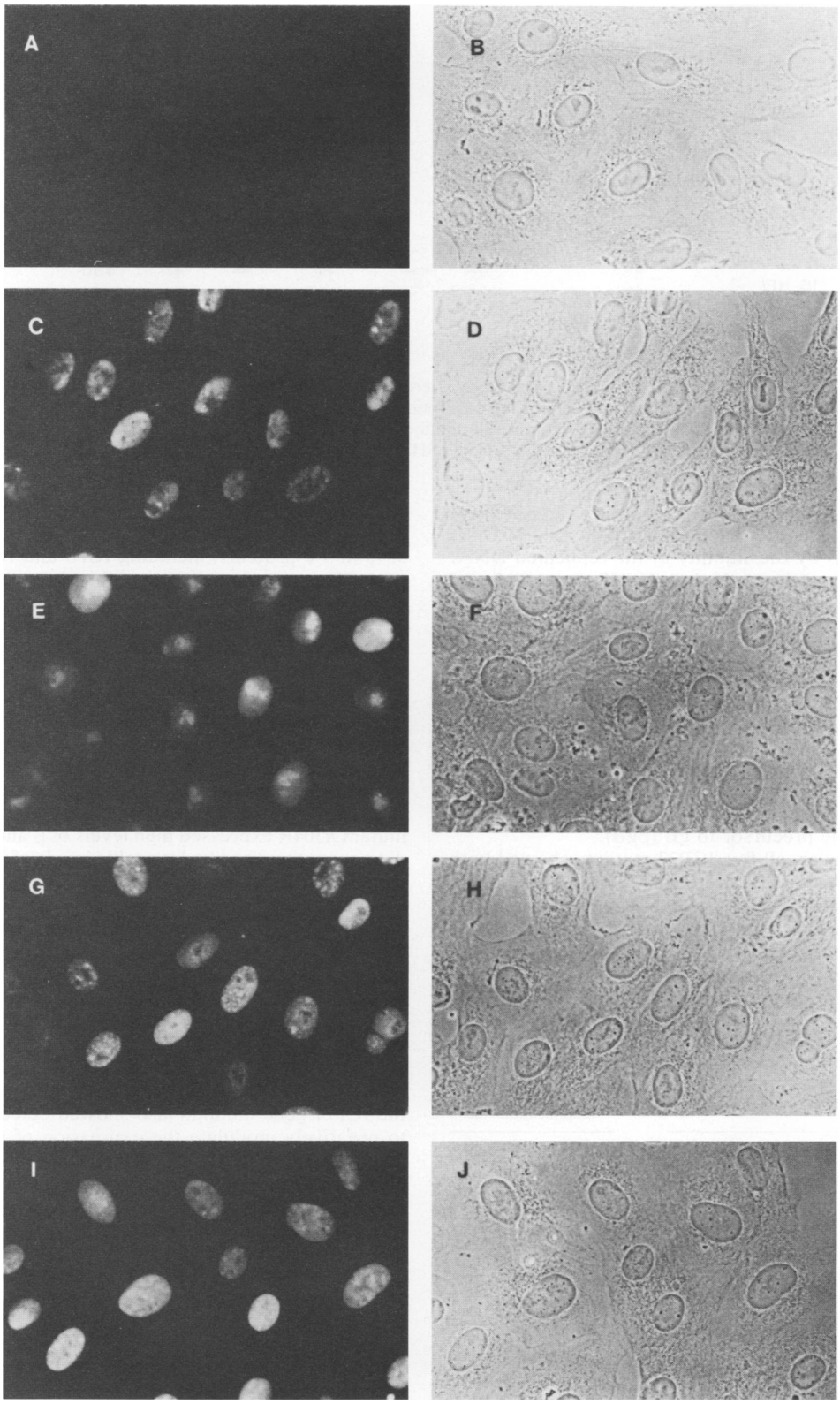

FIG. 4. Cellular localization of mutant ICP27 molecules. Vero cells were mock-infected (A and B) or infected with WT virus (C and D), *n*263R (E and F), *n*406R (G and H), or *n*504R (I and J). At 4 h p.i., the cells were fixed and processed for immunofluorescence microscopy, using the antibody H1113. Panels A, C, E, G, and I are immunofluorescence micrographs, and panels B, D, F, H, and J are the corresponding phase-contrast micrographs.

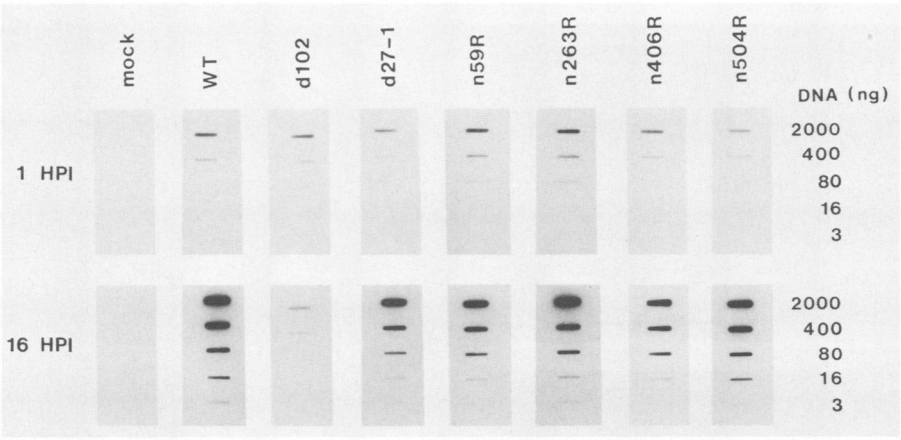

FIG. 5. Ability of viral ICP27 mutants to replicate viral DNA. Vero cells were mock-infected or infected with WT HSV-1 or various mutants. Total cell DNA was prepared immediately after viral adsorption (1 h p.i.) or near the end of the infectious cycle (16 h p.i.). Equal amounts of each DNA sample were subjected to fivefold serial dilutions, and the dilutions were applied to a nitrocellulose filter, using a slot-blot manifold. The filter was probed with ^{32}P-labeled DNA from pSHZ, a plasmid containing an HSV-1 DNA insert (29); an autoradiogram of the blot is shown.

in infected cells, a quantity determined by the rate of DNA synthesis as well as by the stability of the replicated DNA.

Patterns of viral protein synthesis in ICP27 mutant-infected cells. To determine the effect of the ICP27 defects on viral gene expression, we analyzed viral protein synthesis in mutant virus-infected cells. Mock- or HSV-1-infected Vero cells were pulse-labeled with [^{35}S]methionine at 3, 6, or 9 h p.i. The labeled proteins were separated by SDS-PAGE and visualized by autoradiography (Fig. 6). With the exception of *n*406R, the ICP27 gene mutants at 3 and 6 h p.i. showed patterns of protein synthesis that were qualitatively similar to the WT pattern. The mutant *n*406R, however, failed to fully induce the synthesis of several proteins, including ICP6, ICP8, and the precursor to gB (pgB).

At 9 h p.i., however, all five ICP27 mutants showed both quantitative and qualitative differences in viral protein synthesis compared with the WT. The five mutants fell into four phenotypic classes with respect to viral protein synthesis at late times. The phenotypes of *d*27-1 and *n*59R were indistinguishable in this experiment and in several repeat experiments. Both of these mutants expressed high levels of most β proteins but expressed lower levels, relative to WT, of several γ-1 proteins, including ICP5 and ICP25 (Fig. 6, 9 h p.i.). The mutant *n*263R exhibited a pattern of protein synthesis at 9 h p.i. that was very similar to that of *d*27-1 and *n*59R, but this mutant expressed slightly more of several γ-1 proteins. The mutant *n*406R had an unusual phenotype with regard to viral protein synthesis that was quite evident at 9 h p.i. Cells infected with *n*406R expressed greatly reduced levels of many viral proteins, including ICP6, ICP8, and pgB. The reduction of viral gene expression in *n*406-infected cells, however, was not a general effect because they made detectably greater amounts of the γ-1 proteins ICP5 and ICP25 at 9 h p.i. than did *d*27-1-infected cells. Finally, the mutant *n*504R expressed high levels of β and γ-1 proteins but did not express readily detectable levels of γ-2 proteins, such as ICP1/2 and ICP15.

TABLE 2. Viral DNA replication by ICP27 mutants

Virus	DNA amplification[a] in:		% of WT amplification[b] in:	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
KOS1.1 (WT)	80	142	100	100
*d*102	0.5	0.4		
*d*27-1	18	11	23	8
*n*59R	15	22	19	15
*n*263R	17	9	21	6
*n*406	30	23	38	16
*n*504	98	121	123	85

[a] The numbers shown are the ratio of the amount of HSV-1 DNA in infected Vero cells at 16 h p.i. to the amount present at 1 h p.i. The DNA was detected as shown in Fig. 5 and quantitated as described in Materials and Methods (experiment 1 corresponds to Fig. 5, while experiment 2 was a separate experiment). The probe in experiment 1 was pSHZ, and the probe in experiment 2 was pRB3441.

[b] The amount of DNA amplification by the WT virus in each experiment has been normalized to 100%, and the other values are expressed relative to this value. Because *d*102 showed a decrease in the amount of DNA during infection, a percentage value was not determined.

At 9 h p.i., WT virus-infected cells showed a down regulation of the expression of the α proteins ICP4 and ICP27 (Fig. 6). All five ICP27 gene mutants, however, were defective at negatively regulating the expression of ICP4. In addition, *n*504R-infected cells failed to turn off expression of the ICP27 polypeptide (because none of the other mutants encodes a protein that comigrates with WT ICP27, this was the only case in which a direct comparison of ICP27 protein synthesis rates was possible). With the exception of *n*406R, the ICP27 gene mutants also showed defects in their ability to negatively modulate the expression of many β proteins, including ICP6 and ICP8. These results suggest that ICP27 has a negative regulatory effect on the expression of α and β genes.

We also determined whether the defects in viral protein synthesis observed in the infections with mutant viruses could be corrected by expression of the WT ICP27 protein in V27 cells. Vero or V27 cells were infected in parallel with WT virus or with one of the ICP27 gene mutants, and the infected cells were pulse-labeled with [^{35}S]methionine at 15 h p.i. The labeled proteins were analyzed by SDS-PAGE and autoradiography (Fig. 7). The patterns of protein synthesis observed at 15 h p.i. in Vero cells were consistent with the patterns observed in the previous experiment at 9 h p.i. However, when the infections involving mutant viruses were carried out in V27 cells, the WT pattern of protein synthesis was largely restored. These results indicate that the major

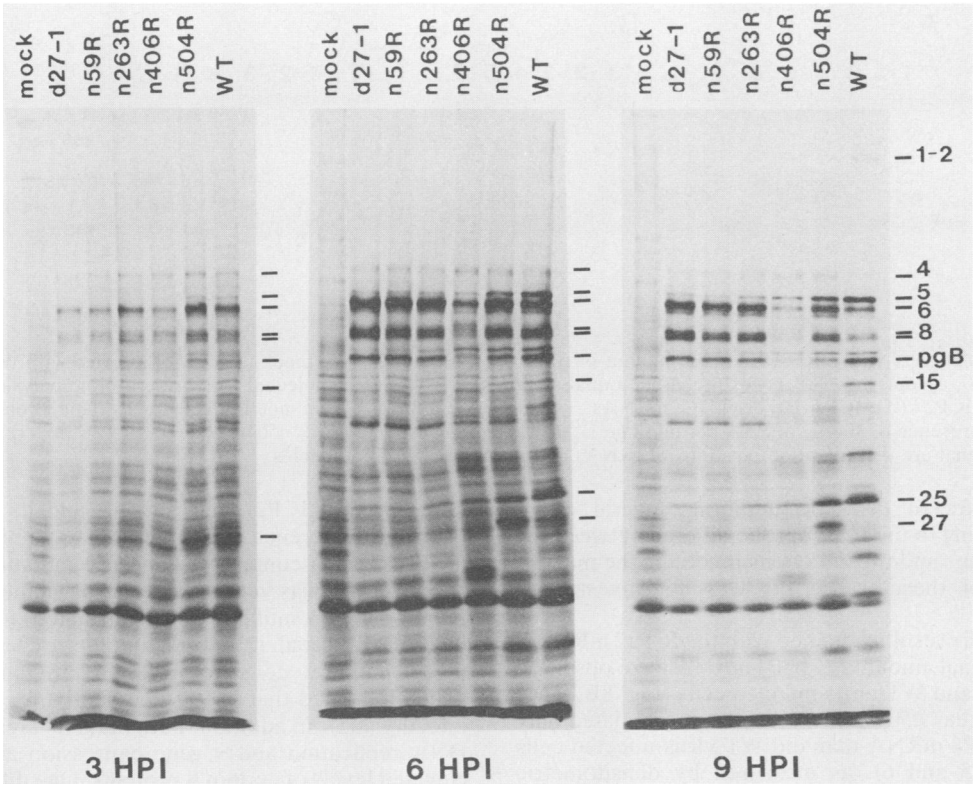

FIG. 6. Protein synthesis in ICP27 mutant-infected cells. Vero cells were mock-infected or infected with WT HSV-1 or various ICP27 mutants. At 3, 6, or 9 h p.i., the cells were labeled for 30 min with [^{35}S]methionine and then harvested. Equal fractions of each cell lysate were subjected to SDS-PAGE and autoradiography. Shown at the right of each panel are the positions of migration of several HSV-1 proteins.

defects in protein synthesis observed in Vero cells are in fact due to defects in the ICP27 gene and not due to any potential secondary mutations which map outside of the ICP27 gene.

Accumulation of viral mRNAs in ICP27 mutant-infected cells. The experiments described above indicated that all five ICP27 mutants were defective for both the down regulation of the α protein ICP4 and the induction of γ-2 proteins. In addition, the phenotype of the mutant *n*504R suggested that ICP27 may act to negatively regulate its own expression. To address the mechanisms by which ICP27 affects the expression of the ICP4, ICP27, and γ-2 genes, we measured the steady-state level of viral mRNAs by Northern blot analysis. Vero cells were mock-infected or infected with WT virus, *n*59R, *d*27-1, or *n*504R. The WT virus infections were carried out in the presence or absence of phosphonoacetic acid (PAA), a specific inhibitor of HSV-1 viral DNA synthesis. Cytoplasmic RNA was isolated from the infected cells at 9 h p.i. Equal amounts of RNA were subjected to electrophoresis in formaldehyde-agarose gels and transferred to nylon filters. The filters were probed with radiolabeled DNAs specific for the ICP27 (Fig. 8A), ICP4 (Fig. 8B), or gC (Fig. 8C) mRNA.

No ICP27-specific mRNAs were detected in mock- or *d*27-1-infected cells (Fig. 8A, lanes 2 and 3). The latter result was expected because the deletion in *d*27-1 removes the ICP27 gene promoter as well as 80% of the coding region. RNA isolated from *n*59R- or *n*504R-infected cells (Fig. 8A, lanes 4 and 5) contained two- to threefold more 2.0-kb ICP27 mRNA than did RNA from WT virus-infected cells (Fig. 8A, lane 6), as shown by densitometric analysis of the autoradiograms. This result was qualitatively consistent with the elevated levels of ICP27 protein synthesis observed at 9 h

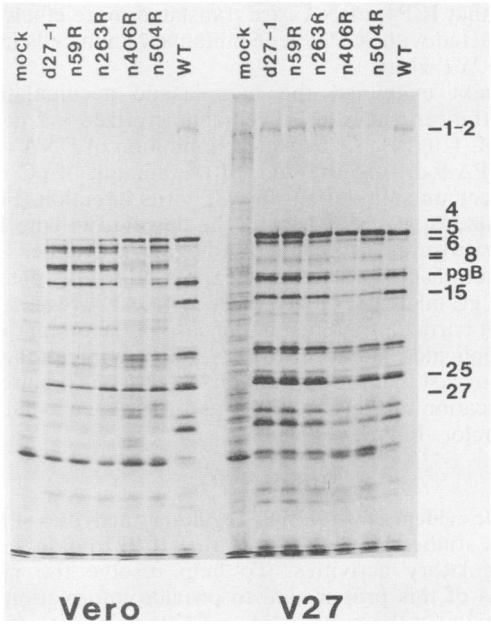

FIG. 7. Complementation of protein synthesis defects in V27 cells. Vero or V27 cells were mock-infected or infected with WT HSV-1 or various mutants. The cells were labeled for 30 min at 15 h p.i. with [^{35}S]methionine, and protein synthesis was analyzed as described in the legend to Fig. 6. Shown at the right are the positions of migration of several HSV-1 proteins.

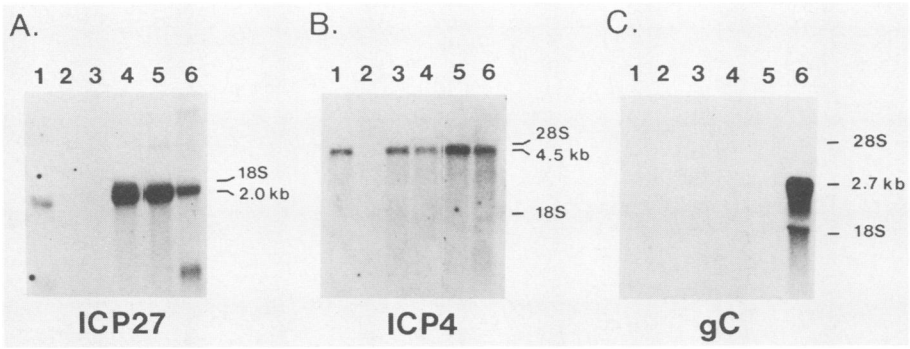

FIG. 8. Accumulation of viral mRNAs in ICP27 mutant-infected cells. Vero cells were mock-infected or infected with WT HSV-1 or ICP27 mutants. At 9 h p.i., cytoplasmic RNA was prepared. Equal amounts of RNA were subjected to Northern blot analysis using ^{32}P-labeled probes specific for ICP27 (A), ICP4 (B), or gC (C) mRNAs. The lanes contain RNA from mock-infected cells (lane 2) or cells infected with WT HSV-1 in the presence of 400 μg of PAA per ml (lane 1), *d*27-1 (lane 3), *n*59R (lane 4), *n*504R (lane 5), or WT HSV-1 (lane 6). Shown to the right of each panel are the migration positions of rRNAs and major viral mRNA species.

p.i. in *n*504R-infected cells. In addition, several minor mRNAs hybridizing to the ICP27 probe differed between the WT virus infections and the infections involving the mutant, but the origin of these transcripts was not investigated further.

In contrast to the results obtained with the ICP27 mRNAs, approximately equal amounts of ICP4 mRNA were observed in *d*27-1-, *n*59R-, and WT virus-infected cells (Fig. 8B, lanes 3, 4, and 6), whereas *n*504R-infected cells accumulated only 1.6-fold more ICP4 mRNA than did WT virus-infected cells (Fig. 8B, lanes 5 and 6), as measured by densitometric analysis. These results were somewhat unexpected because little or no ICP4 protein synthesis was detected at 9 h p.i. in WT virus-infected cells, but ICP4 synthesis was readily detected in cells infected with the ICP27 gene mutants (Fig. 6, 9 h p.i.). Therefore, the level of ICP4 protein synthesis occurring at 9 h p.i. in the various infections did not reflect the level of cytoplasmic ICP4 transcripts. These results suggest that ICP4 mRNAs are translated more efficiently in cells infected with ICP27 gene mutants than in cells infected with the WT virus.

We next examined the cytoplasmic accumulation of mRNA transcribed from a well-characterized γ-2 gene encoding gC (10, 21). As expected, inhibition of DNA replication by PAA drastically reduced the amount of gC mRNA which accumulated during the WT virus infection (Fig. 8C, lanes 1 and 6; gC mRNA could be detected in lane 1 upon longer exposures of the autoradiogram). Neither *d*27-1-, *n*59R-, nor *n*504R-infected cells expressed readily detectable levels of gC mRNA (Fig. 8C, lanes 3, 4, and 5, respectively). This is of particular interest in the case of the mutant *n*504R, which replicated WT levels of DNA during infection. These results suggest that the expression of γ-2 genes requires both the replication of the viral DNA and a virus-encoded *trans*-acting factor, ICP27.

DISCUSSION

Genetic evidence for multiple regulatory activities of ICP27. Previous studies have suggested that ICP27 possesses multiple regulatory activities. To help resolve the multiple functions of this protein and to provide information about what regions of the polypeptide mediate its effects, we have constructed several viral mutants containing nonsense mutations which encode truncated forms of ICP27. We also isolated *d*27-1, an HSV-1 mutant which contains a deletion in the ICP27 gene that removes the promoter as well as 80% of the coding region.

*d*27-1 is an ICP27 null mutant, and its phenotype thus provides a reference point for understanding the phenotypes of the mutants containing nonsense mutations. The phenotype of *d*27-1 was very similar, if not identical, to that of an ICP27 deletion mutant recently isolated and characterized by McCarthy et al. (29). Both mutants are unable to replicate in cell culture, consistent with a previous genetic study which indicated that ICP27 is essential for the HSV-1 lytic infection (41). In addition, both mutants are deficient in viral DNA replication and γ gene expression and both express elevated levels of certain β proteins. One difference between our results and those of McCarthy et al., however, concerns the expression of the polypeptide ICP4. We found that cells infected with *d*27-1 synthesize higher levels of ICP4 at late times than do cells infected with the WT virus, while McCarthy et al. detected no effect of the ICP27 gene defect on ICP4 expression.

The comparison of the phenotypes of the viral mutants carrying nonsense mutations with the WT and null phenotypes has allowed us to define the following regulatory effects of ICP27: (i) stimulation of γ-1 gene expression, (ii) induction of γ-2 gene expression, (iii) negative regulation of the expression of certain α and β genes, and (iv) stimulation of viral DNA replication. As we discuss below, our results demonstrate that certain of these activities are genetically separable.

Stimulation of γ-1 gene expression by ICP27. Cells infected with the ICP27 null mutant, *d*27-1, were unable to fully activate expression of most γ-1 genes. Thus, ICP27 carries out an activity in infected cells that enhances γ-1 gene expression. Amino-terminal portions of ICP27 appeared to be able to partially induce γ-1 gene expression, because *n*263R- and *n*406R-infected cells synthesized more of several γ-1 proteins, such as ICP5, late in infection than did *d*27-1- or *n*59R-infected cells. However, the mutants *n*263R and *n*406R did not appear to replicate significantly more viral DNA over the course of infection than either *d*27-1 or *n*59R. Therefore, the increase in the expression of the γ-1 proteins appears to be due to an effect of ICP27 on γ-1 gene expression other than stimulation of viral DNA replication. The mutant *n*504R expressed WT levels of γ-1 proteins. However, because this mutant replicated more viral DNA than did *n*406R, it is not clear whether the increased expression of γ-1 genes is due to an increased transactivation function of the *n*504R polypeptide or due to increased levels of viral DNA replication.

The mechanism by which ICP27 stimulates expression of the γ-1 genes is not known. It is worth noting that when

infections were carried out in the presence of PAA to inhibit viral DNA replication, comparable levels of γ-1 proteins were expressed in *d*27-1-infected and WT virus-infected cells (data not shown). This observation suggests that the γ-1 transactivation function of ICP27 is active only in cells that are undergoing or have undergone viral DNA replication.

Induction of γ-2 gene expression by ICP27. Cells infected with *n*504R replicated WT levels of viral DNA and expressed WT levels of γ-1 proteins but failed to induce synthesis of γ-2 proteins. These results provide strong evidence that ICP27 possesses a second transactivation function which is distinct from its ability to stimulate γ-1 genes. The carboxyl-terminal region of ICP27 is required for this second activity of ICP27 but is not required for γ-1 gene stimulation. For the γ-2 gC gene, we found that *n*504R-infected cells failed to express readily detectable levels of cytoplasmic gC mRNA. Thus, our results are consistent with a role for ICP27 in the transcriptional induction of the γ-2 genes.

Previous studies have implicated ICP4 in the activation of γ-2 genes, most likely at the level of transcription (6, 8, 51). In addition to ICP27 and ICP4, the expression of the γ-2 mRNAs requires replication of the viral DNA template (14, 18, 20, 23). Mavromara-Nazos and Roizman have used superinfection experiments to study the role of DNA replication in γ-2 gene expression. They demonstrated that *trans*-acting factors present in an HSV-1-infected cell that has replicated viral DNA are not sufficient to activate the γ-2 genes of a superinfecting genome (28). Rather, the superinfecting genome itself needs to undergo viral DNA replication before its γ-2 genes are expressed. Thus, γ-2 gene expression appears to require a modification of the viral DNA template, as well as two virus-encoded *trans*-acting factors, ICP27 and ICP4. More work is necessary to understand how these *cis*- and *trans*-acting factors interact to determine the expression of these genes. *n*504R will probably be a valuable tool in these studies, because the infection of cells with this mutant provides a situation in which the replication of the viral DNA can be uncoupled from the expression of the γ-2 genes.

Negative regulation of α and β gene expression by ICP27. Cells infected with any one of the five ICP27 mutants synthesized substantially higher levels of the α protein ICP4 at late times after infection than did WT virus-infected cells. Furthermore, *n*504R-infected cells failed to down regulate ICP27 synthesis late in infection (this was the only case in which direct comparison of ICP27 protein synthesis rates was possible). Thus, our results suggest that ICP27 normally plays a role, either directly or indirectly, in down regulating the expression of α genes. McCarthy et al. previously noted that cells infected with an ICP27 deletion mutant overexpressed many β proteins, including ICP6 and ICP8 (29). We also observed overexpression of β proteins in cells infected with our deletion mutant, *d*27-1. In addition, with the exception of *n*406R, the viruses containing nonsense mutations that we isolated also showed overexpression of β proteins. Because *n*406R was unable to efficiently induce expression of most β genes, it is impossible to say whether this mutant is deficient at down regulating these genes.

The overexpression of α genes in ICP27 mutant-infected cells was investigated further by Northern blot analysis. Cells infected with either *n*59R or *n*504R expressed significantly higher levels of ICP27 mRNA than did WT virus-infected cells. This was consistent, at least qualitatively, with the higher rates of ICP27 protein synthesis seen in *n*504R-infected cells. Thus, ICP27 appears to have an autoregulatory function. A study of the ICP27 promoter in a transient-expression system has also provided evidence for ICP27 autoregulation (12). In contrast to the results with ICP27 mRNA, ICP4 mRNA levels did not differ significantly between WT virus- and ICP27 gene mutant-infected cells. This was interesting because cells infected with these mutants synthesized substantially higher levels of ICP4 protein at this same time point. These results suggest that the WT ICP27 may act at some level to negatively regulate the translation of ICP4 mRNA and that ICP27 may exert two regulatory effects on α gene expression.

The phenotype of the mutant *n*406R provides additional evidence for a negative regulatory activity of ICP27. Cells infected with *n*406R were unable to induce efficient synthesis of many polypeptides, including ICP6 (β), ICP8 (β), and pgB (β or γ-1). Because these genes were expressed well in cells infected with the null mutant *d*27-1, it would seem that the *n*406R protein carries out an activity in infected cells that interferes with expression of these genes. The interference effect, however, is not a general one because γ-1 genes such as those for ICP5 and ICP25 were expressed at higher levels in *n*406R-infected cells than in *d*27-1-infected cells. The phenotype of *n*406R appears similar in many respects to those of certain ICP27 gene *ts* mutants, which at the nonpermissive temperature show reduced expression of several gene products, including ICP6, ICP8, and pgB (39, 41).

Further studies are required to determine what region(s) of the ICP27 polypeptide mediates negative regulation. The phenotype of *n*504R would suggest that an intact carboxyl terminus of ICP27 is required for efficient negative regulation of α and β genes. However, in a transient-expression system, the amino-terminal 406 amino acid residues are sufficient for negative regulation (40). Furthermore, the phenotype of the *n*406R mutant in this study suggests that this truncated polypeptide can interfere with gene expression in infected cells.

Stimulation of viral DNA replication by ICP27. It is clear from our results and from those of McCarthy et al. (29) that ICP27 is not absolutely required for viral DNA replication but is required for WT levels of viral DNA replication. ICP27 may enhance the accumulation of viral DNA either by increasing the rate of viral DNA synthesis or by increasing the stability of replicated DNA. There are at least two general mechanisms by which ICP27 may exert these effects. First, the ability of ICP27 to stimulate viral DNA replication might be an indirect effect of its ability to stimulate gene expression. ICP27 might increase the expression of a viral protein (or proteins) which enhances DNA synthesis or its stability. Alternatively, the ICP27 function which stimulates DNA accumulation may be independent of its ability to modulate gene expression, perhaps by functioning in some more direct role in the DNA replication process.

Our results indicate that amino acid residues 407 to 504 of ICP27 are critical for this function because the *n*406R mutant is deficient for viral DNA replication while the *n*504R mutant is not. The mutants *n*263R and *n*406R did not appear to replicate significantly more viral DNA than did *d*27-1, but they did express detectably more γ-1 proteins. Thus, we favor the idea that these two functions of ICP27, i.e., its ability to stimulate γ-1 gene expression and its ability to stimulate viral DNA replication, are genetically separable. However, further experiments are required to prove this point conclusively.

Regulation of the gB gene. A possible exception to our generalizations concerning the response of HSV-1 genes to ICP27 is the gB gene. The generalizations hold true if the gB gene is considered to be a β gene rather than a γ-1 gene.

However, various studies have described it as either a β (19, 37) or a γ-1 (21, 52) gene. This gene may in fact have regulatory characteristics which do not allow it to fit neatly into either classification.

We found previously that the gB promoter can be transactivated by ICP27 in a transient-expression assay (39). In addition, we found that the gB gene is expressed poorly in cells infected with ICP27 *ts* mutants at the nonpermissive temperature. Thus, we proposed that ICP27 is a specific transactivator of the gB gene. However, our present studies and those of McCarthy et al. (29) clearly demonstrate that expression of this gene can be induced to WT levels in infected cells in the absence of ICP27. It is of interest that the mutant *n*406R is similar to the *ts* mutants in its inability to induce normal expression of the gB gene. Thus, some altered ICP27 molecules may interfere with the expression of this gene in infected cells.

Genetic system for studying ICP27. Previously, we used transient-expression assays to compare the regulatory effects of WT and mutant ICP27 genes on several target HSV-1 promoters (40). The results of that study indicated that ICP27 possesses genetically separable positive and negative regulatory activities. In the present study, those mutant alleles were introduced into the viral chromosome and the phenotypes of the resulting viruses were studied. In some cases, these phenotypes are not easily correlated with the properties of the mutant genes when they were expressed in transfected cells. For example, the viral mutants *n*406R and *n*504R differed dramatically in their abilities to express viral genes, but the two mutant genes appeared to have identical regulatory properties in transfected cells. We can propose two explanations for the difficulty in extrapolating the results of transient-expression experiments to the results obtained by using infected cells. First, the ability to examine the effect of ICP27 on the expression of a large number of genes during the viral infection may reveal activities which are not apparent in transient-expression experiments, in which fewer target genes are studied. Second, the regulation of viral genes in the infected cell differs inherently from that in the uninfected cell because of the occurrence of processes such as viral DNA replication and because of the interaction of multiple viral regulatory proteins with each other and with cellular proteins.

ICP27 seems to be similar to several other viral regulatory proteins in possessing multiple domains which carry out distinct activities. Our studies, and those of others (17), have begun to localize the functional regions of the polypeptide, but much work needs to be done. The genetic system we describe here can be used to isolate a large number of viral mutants encoding specifically altered forms of ICP27. The characterization of such mutants should provide a useful approach both for mechanistic studies of ICP27 and for the detailed mapping of its functional domains.

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