The Conserved Carboxyl-Terminal Half of Herpes Simplex Virus Type 1 Regulatory Protein ICP27 Is Dispensable for Viral Growth in the Presence of Compensatory Mutations

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ICP27 is an essential herpes simplex virus type 1 (HSV-1) immediate-early protein that regulates viral gene expression by poorly characterized mechanisms. Previous data suggest that its carboxyl (C)-terminal portion is absolutely required for productive viral infection. In this study, we isolated M16R, a second-site revertant of a viral ICP27 C-terminal mutant. M16R harbors an intragenic reversion, as demonstrated by the fact that its cloned ICP27 allele can complement the growth of an HSV-1 ICP27 deletion mutant. DNA sequencing demonstrated that the intragenic reversion is a frameshift alteration in a homopolymeric run of C residues at codons 215 to 217. This results in the predicted expression of a truncated, 289-residue molecule bearing 72 novel C-terminal residues derived from the +1 reading frame. Consistent with this, M16R expresses an ICP27-related molecule of the predicted size in the nuclei of infected cells. Transfection-based viral complementation assays confirmed that the truncated, frameshifted protein can partially substitute for ICP27 in the context of viral infection. Surprisingly, its novel C-terminal residues are required for this activity. To see if the frameshift mutation is all that is required for M16R's viability, we re-engineered the M16R ICP27 allele and inserted it into a new viral background, creating the HSV-1 mutant M16exC. An additional mutant, *exCd305*, was constructed which possesses the frameshift in the context of an ICP27 gene with the C terminus deleted. We found that both M16exC and *exCd305* are nonviable in Vero cells, suggesting that one or more extragenic mutations are also required for the viability of M16R. Consistent with this interpretation, we isolated two viable derivatives of *exCd305* which grow productively in Vero cells despite being incapable of encoding the C-terminal portion of ICP27. Studies of viral DNA synthesis in mutant-infected cells indicated that the truncated, frameshifted ICP27 protein can enhance viral DNA replication. In summary, our results demonstrate that the C-terminal portion of ICP27, conserved widely in herpesviruses and previously believed to be absolutely essential, is dispensable for HSV-1 lytic replication in the presence of compensatory genomic mutations.

Herpes simplex virus type 1 (HSV-1), one of the most intensively studied and best-characterized herpesviruses, serves as a prototype for understanding how these important DNA viruses replicate in their host eukaryotic cells. The HSV-1 genome is composed of ~152,000 bp of double-stranded DNA and encodes approximately 80 proteins (for a review, see reference 32). During lytic infection, the viral genes are transcribed in the cell nucleus by cellular RNA polymerase II. However, despite its dependence on the host cell, HSV-1 is able to impose tight regulatory control on its genes such that they are expressed in a coordinately activated cascade consisting of three temporal phases known as the immediate-early (IE, also known as α), delayed-early (DE, also known as β), and late (L, also known as γ) phases (reviewed in reference 40).

Approximately half of the HSV-1 genes can be considered essential in that they are absolutely required for productive infection of cultured cells (32). One gene which is clearly in this category is that encoding infected cell protein ICP27. ICP27 is a 512-residue IE protein which is localized predominantly to infected cell nuclei. Viral mutants that make temperature-sensitive forms of ICP27 fail to replicate at the nonpermissive temperature (33). Furthermore, mutants with the ICP27 gene

deleted are completely nonviable in tissue culture but can be propagated in engineered cell lines which possess a stably transfected ICP27 gene (14, 27).

Characterization of a number of viral ICP27 mutants has indicated that ICP27 performs several regulatory functions during viral lytic infection. First, it is a critical activator of viral gene expression. In the absence of functional ICP27, many DE and L viral genes are not expressed efficiently as mRNAs or proteins (14, 15, 27, 29, 33, 39). Second, ICP27 represses viral IE and DE genes at late times after infection (14, 27, 33). Third, ICP27 contributes to the shutoff of host gene expression, as both cellular protein synthesis and mRNA levels are elevated in ICP27 mutant infections compared to wild-type (WT) HSV-1 infection (8, 33, 38). Fourth, ICP27 stimulates viral DNA replication by approximately 10-fold (14, 27). This function is likely attributable to ICP27's ability to transactivate several DE genes which encode viral DNA replication factors (15, 39). Fifth, in human cells, ICP27 prevents virus-induced apoptosis (2). It is not known whether this function results from ICP27's ability to modulate viral or cellular genes.

Despite its many potent regulatory effects, the molecular mechanism(s) by which ICP27 carries out its activities is largely uncharacterized. However, considerable evidence indicates that at least some of its effects on gene expression occur at the posttranscriptional level. A variety of studies have demonstrated that under certain experimental conditions, ICP27 can (i) modulate the efficiency of pre-mRNA polyadenylation (15, 16, 36), (ii) alter the intranuclear distribution of pre-mRNA

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splicing factors (23, 35), (iii) inhibit pre-mRNA splicing (10), (iv) increase the stability of specific cellular mRNAs (4), and (v) inhibit the nuclear export of intron-containing viral transcripts (25). Furthermore, ICP27 has some of the characteristics of a posttranscriptional regulatory protein. It is able to bind to RNA both *in vitro* (4, 12, 20) and *in vivo* (34), although it is unclear whether it recognizes a specific target sequence. Moreover, similar to some other posttranscriptional regulators, ICP27 shuttles continuously between the nucleus and the cytoplasm (19, 24, 34, 38). Recently, it has been suggested that ICP27 has a role in the nuclear export of intronless viral mRNAs (34, 38).

The functional domains of ICP27 have not been clearly delineated, but numerous studies suggest that the C-terminal half of the protein (from approximately residue 260 to the C terminus at residue 512) is especially critical. Mutagenesis experiments have indicated that stop codon insertions, in-frame linker insertions, in-frame deletions, or alterations of selected codons in the C-terminal half of the ICP27 gene all disrupt the protein's ability to modulate reporter genes in transfection assays (9, 17, 31). Moreover, many C-terminal mutations are lethal when introduced into recombinant viruses (17, 27, 28) or when tested in plasmid-based viral complementation assays (17, 31). Interestingly, genomic sequence analyses have suggested that all mammalian and avian herpesviruses encode a protein with homology to the C-terminal ~200 residues of ICP27 (3, 4, 22). In contrast, the N-terminal portion of ICP27 is not closely conserved among herpesviruses. Taken together, these studies suggest that the C-terminal half of ICP27 has a fundamental role in one or more of its essential regulatory functions.

This report describes a genetic study which was initiated to focus on the C-terminal region of ICP27. We utilized the HSV-1 ICP27 mutant M16, which fails to replicate in cultured cells due to an amino acid alteration at residue 488, near ICP27's C terminus (28). Our goal was to isolate second-site revertants with the idea that such mutants might provide clues to ICP27's tertiary structure and possibly identify viral proteins that interact with the C terminus. We were successful in isolating a viable second-site revertant, designated M16R. However, analysis of M16R led to a very surprising finding: this mutant harbors a frameshift mutation in the N-terminal half of the ICP27 gene and thus does not apparently express the C-terminal half of ICP27. Further analysis of M16R and other, similar mutants has led us to the quite unexpected conclusion that the C-terminal half of ICP27 is not absolutely required for the lytic growth of HSV-1 in cultured cells. This finding provides novel insight into ICP27's functions and functional domains.

MATERIALS AND METHODS

Cells and viruses. Vero cells, obtained from the American Type Culture Collection, and V27 cells, Vero cell derivatives which contain a stably transfected copy of the ICP27 gene (27), were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 5% FBS plus geneticin (G418; Gibco-BRL) at 100 µg/ml, respectively. WT HSV-1 strain KOS1.1 (11) was propagated in Vero cells. The HSV-1 ICP27 mutants *d27-1* (27) and M16 (28) were propagated in V27 cells. The isolation and propagation of other HSV-1 mutants are described below.

For isolation of M16R, six M16 plaques were isolated in V27 cells. These were subjected to three rounds of freeze-thawing, and a portion of each was passaged in Vero cells growing in 25-cm² flasks. After 48 h, the cells were subjected to freeze-thawing and the resulting lysates were passaged on 25-cm² flasks of Vero cells. Cytopathic effects (CPE) indicative of HSV-1 infection appeared in one flask 3 days postinfection. A viral isolate from this flask was plaque purified one time in Vero cells and designated M16R. Restriction analysis of a PCR fragment derived from M16R DNA confirmed that the virus retains the *Xho*I site which marks the original M16 mutation. M16R was further plaque purified in Vero

cells, and a low-titer stock was prepared in Vero cells. High-titer stocks were prepared in V27 cells.

The HSV-1 ICP27 mutants M16exC, *exCd305*, and *n217d* were engineered by *in vivo* homologous recombination using a previously described protocol (27, 28), with the exception that transfections were carried out using the Lipofectamine reagent (Gibco-BRL) as described below. For each engineered virus, two viral isolates were obtained from independent transfections. The ICP27 gene structures of all isolates were confirmed by extensive PCR analysis.

For isolation of R1exCd305 and R2exCd305, four *exCd305* plaques were isolated in V27 cells. These were amplified once by passage in V27 cells growing in 3.8-cm² wells of a 12-well plate. At 6 days postinfection, virus was released by freeze-thawing and a portion of each lysate was passaged in 75-cm² flasks of Vero cells for 2 days. Infected cell lysates were prepared by freeze-thawing and passaged sequentially up to three times in 25-cm² flasks of Vero cells for 4 to 5 days per passage. By passage three, three of the four lysates had yielded cultures which exhibited characteristic HSV-1 CPE. DNA was isolated from the positive flasks, and the ICP27 gene was amplified from the DNA samples by PCR. One sample yielded an ICP27 gene PCR fragment with no deletion, suggesting that the virus present had a WT ICP27 allele, possibly obtained by recombination of *exCd305* with the stably transfected ICP27 gene of V27 cells. However, the other two DNA samples yielded ICP27 gene PCR products which still possessed the *exCd305* deletion, indicating the presence of second-site revertants. Viral isolates from each of the two positive lysates were plaque purified three times in V27 cells and designated R1exCd305 and R2exCd305. Mutants stocks were prepared in V27 cells.

Plasmid constructs. All plasmids used for complementation experiments possess the ICP27 gene within a *Bam*HI-*Sac*I HSV-1 fragment. In the case of the WT virus, this fragment is 2.4 kb. The plasmids were generated by the following manipulations. Plasmids p27 and pM1627 contain the ICP27 alleles of WT HSV-1 (strain KOS1.1) and M16, respectively, and were constructed by cloning the 2.4-kb *Bam*HI-*Sac*I fragments from pM27 (28) and pM16 (28) into pUC19. Plasmid pM16R was created by cloning the 2.4-kb *Bam*HI-*Sac*I fragment of M16R, isolated from viral genomic DNA, into pUC19. Plasmids pWTexC and pM16exC are derivatives of plasmid pM27exC. pM27exC was constructed by oligonucleotide-directed mutagenesis (Altered Sites system; Promega) starting from pM27. Relative to pM27, it possesses a single base pair insertion at codons 215 to 217, converting the run of eight coding strand cytosines to nine. pWTexC was created by three-fragment ligation involving the 2.7-kb *Sac*I-*Bam*HI fragment of pUC19, the 1.2-kb *Bam*HI-*Sac*I ICP27 gene fragment from pM27exC, and the 1.2-kb *Sal*I-*Sac*I ICP27 allele fragment from p27. pM16exC was engineered using the same strategy, except that the 1.2-kb *Sac*I-*Sac*I fragment was derived from pM1627. To construct plasmid pexCn406, a 12-bp *Nhe*I linker containing stop codons in all three reading frames (New England Biolabs) was cloned into the *Stu*I site of pWTexC. pWTn406 was constructed by a three-fragment ligation involving the 2.7-kb *Sac*I-*Bam*HI fragment from pUC19, the *Bam*HI-*Sal*I fragment from p27, and the *Sal*I-*Sac*I fragment from pexCn406. Plasmids pexCd305 and pn217d possess a C-terminal deletion in the ICP27 coding region. To create this deletion, plasmid pPsd10-11 (V. Leong and S. A. Rice, unpublished data, 2000), was utilized. This plasmid contains an ICP27 allele which has an engineered *Xho*I site at codon 304 and lacks codons 306 to 339. The C-terminal deletion was engineered by digesting pPsd10-11 with *Xho*I and *Eco*NI, which cuts at the 3' end of the ICP27 coding region. The 3'-recessed ends were filled in using the large fragment of *Escherichia coli* DNA polymerase I and then ligated together. This gave rise to pPsd305, in which the ICP27 gene is missing codons 306 to 512. Plasmid pexCd305 is the product of three-fragment ligation of the 2.7-kb *Sac*I-*Bam*HI fragment from pUC19, the 1.2-kb *Bam*HI-*Sal*I fragment from pM27exC, and the 0.6-kb *Sal*I-*Sac*I fragment from pPsd305. To create pn217d, oligonucleotide-directed mutagenesis was first used to alter pM27 such that an ochre termination codon was created in the ICP27 coding region at codon 218, resulting in plasmid pMn217d. To generate pn217d, three-fragment ligation was carried out using the 1.2-kb *Bam*HI-*Sal*I fragment of pMn217d, the 0.6-kb *Sal*I-*Sac*I fragment from pPsd305, and the 2.7-kb *Sac*I-*Bam*HI fragment from pUC19. Plasmids pd305R1 and pd305R2 were created by cloning the ICP27 gene-containing *Bam*HI-*Sac*I fragments from the R1exCd305 and R2exCd305 viral DNAs, respectively, into pUC19.

The plasmids employed for recombinant HSV-1 production possess the ICP27 gene on a *Pst*I HSV-1 DNA fragment, which in the case of WT virus is 6.0 kb. The plasmids used to engineer the virus mutants M16exC, *exCd305*, and *n217d* were pPsM16exC, pPsexCd305, and pPsn217d, respectively. They were constructed by cloning the ICP27 gene-containing *Bam*HI-*Sac*I fragments from pM16exC, pexCd305, and pn217d, respectively, into pPs27pd1 (31) in place of the corresponding WT fragment.

The *Bam*HI-*Sac*I fragments of pM16R, pd305R1, and pd305R2 were sequenced in their entirety. In addition, the structures of the engineered ICP27 gene mutations in pMn217d and pM27exC were confirmed by DNA sequencing. Sequencing was performed at the Biochemistry Core DNA facility of the University of Alberta using an Applied Biosystems 373A DNA Sequencer.

Complementation assay. The *d27-1* viral complementation assays were carried out as described previously (29, 31), except that transfections were carried out with 1 µg of plasmid DNA using the Lipofectamine reagent (Gibco-BRL). Lipofectamine transfections for complementation assay and virus constructions were carried out as follows. Purified DNA was mixed with serum-free DMEM to

give a final volume of 200 μ l. Separately, 10 μ l of Lipofectamine reagent per μ g of DNA was mixed with serum-free DMEM to give a volume of 200 μ l. The two mixtures were combined and allowed to sit for 15 to 45 min at room temperature. Mixtures were then made up to a final volume of 2 ml with serum-free DMEM and added to 25-cm² flasks of Vero (for complementation assays) or V27 (for virus construction) cells that had been rinsed with serum-free DMEM. After a 5-h incubation at 37°C, the transfection mixtures were removed and replaced with DMEM containing 5% FBS.

Analysis of mutant viruses. To study the growth of mutant viruses, viral plaque assays and single-cycle yield analyses were performed. For HSV-1 plaque assays, virus stocks were titrated on Vero or V27 monolayers using an overlay of medium 199 (Gibco-BRL) containing 1% heat-inactivated calf serum and 1% pooled normal human serum (ICN). Representative plaques or foci were photographed using an Olympus SC 35 Type 12 camera mounted on an Olympus CK40 microscope. Single-cycle growth assays were carried out as described previously (28, 29). Briefly, parallel cultures of Vero and V27 cells were infected with virus at a multiplicity of infection (MOI) of 10 and treated with a glycine-saline solution (pH 3.0) at 2 h postinfection (hpi) as already described to inactivate unadsorbed virus (5). Following incubation for 24 h, the virus yield was determined by plaque assay of the cell lysates on V27 cells.

An immunofluorescence assay was used to determine the localization of mutant ICP27 proteins. Vero cells growing on coverslips were infected at an MOI of 10. At 4 hpi, these were processed for indirect immunofluorescence assay as described previously (26). Monoclonal antibody H1113 (1) (Goodwin Institute for Cancer Research, Plantation, Fla.), which recognizes residues 109 to 137 of ICP27 (18), was used at a dilution of 1:600. The secondary antibody was tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories Inc., Mississauga, Ontario, Canada) diluted 1:200. The cells were visualized with a Zeiss Axioskop 20 fluorescence microscope equipped with a Plan-Neofluar 63 $\times$ objective lens.

Mutant ICP27 polypeptides were characterized by immunoblotting. Vero cells growing in 25-cm² flasks were infected at an MOI of 10 and harvested at 4 or 6 hpi as described previously (30). Total proteins were separated by sodium dodecyl sulfate (SDS)-15% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 10% skim milk in TBST (1 $\times$ Tris-buffered saline, 0.2% Tween 20), washed twice with TBST, and then probed with a 1:1,000 dilution of H1113 in TBST for 45 min. After washing of the filters twice with TBST, immunoreactive proteins were visualized using a horseradish peroxidase-conjugated secondary antibody diluted 1:3,000 in TBST and an enhanced chemiluminescence system (Amersham).

Viral DNA for PCR and Southern analysis was prepared as follows. Cell monolayers growing in wells of 12-well plates were infected at an MOI of 10. For analysis of viral genome structures, V27 cells were used whereas Vero cells were used for the DNA replication experiments. At various times after infection, the infected cells were lysed by the addition of 400 μ l of 10 mM Tris (pH 8.0)-10 mM EDTA-2% SDS-100 μ g of proteinase K per ml. Following incubation at 37°C for a day, 48 μ l of 3 M sodium acetate (pH 5.2) was added to the lysates, which were then extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). DNA was precipitated with 95% ethanol, resuspended in 10 mM Tris (pH 7.6)-1 mM EDTA, and subjected to RNase A (50 μ g/ml) digestion at 37°C for 1 h.

Southern analyses were carried out as follows. Equal masses of DNA extracted from infected cells were cleaved with *Pst*I and *Sa*I and separated on a 1% agarose gel. Following acid cleavage and alkali denaturation, DNA was transferred to a nylon membrane and hybridized with a ³²P-labeled probe generated by random-primer labeling. Hybridization was carried out in 250 mM sodium phosphate (pH 7.2)-7% SDS-1% bovine serum albumin-1 mM EDTA at 65°C for approximately 12 h. The ICP27 gene N terminus-specific probe was a 510-bp PCR fragment corresponding to ICP27 gene N-terminal codons 90 to 259. The C terminus-specific probe was a 588-bp PCR fragment corresponding to codons 308 to 503. The probe used for analysis of viral DNA replication was a 480-bp PCR fragment corresponding to a sequence at the 5' end of the UL55 gene. This sequence encompasses 55 bp upstream of the UL55 coding region, as well as codons 1 to 142. Membranes were washed twice for 15 min (each time) at 68°C in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS and twice for 15 min (each time) at 68°C in 0.1 $\times$ SSC-0.1% SDS prior to film exposure. Signal quantitation was done by phosphorimage analysis using a Fujix BAS100 bioimaging analyzer with MacBAS imaging software.

RESULTS

Isolation of M16R. As reviewed in the introduction, much previous work suggests that the C-terminal portion of HSV-1 ICP27 is essential for its function. To investigate this important region of the protein, we set out to isolate second-site revertants of M16, an HSV-1 mutant with a C-terminal ICP27 mutation (28). The dinucleotide alteration in M16 changes residue 488 of ICP27 from cysteine to leucine (Fig. 1A and B). As a result, M16 is completely unable to replicate in Vero cells but

FIG. 1. Structures of ICP27 alleles and the proteins they encode. Each line denotes an ICP27 allele used in this study. The bars above the lines represent the encoded ICP27 polypeptide; white bars indicate ICP27 sequences, whereas gray bars denote sequences translated from the +1 reading frame.

can replicate efficiently in V27 cells, which possess a stably transfected ICP27 gene (27). To select revertants, six M16 plaques were generated in V27 cells and used to infect small cultures (approximately 3×10^6 cells) of Vero cells. After 2 days, the cells were disrupted by freeze-thawing and a portion of each lysate was passaged on a new set of Vero cells. These cultures were monitored over several days for the appearance of CPE characteristic of HSV-1 infection. Such CPE were observed in one culture. A virus from this culture was plaque purified in Vero cells and designated M16R. To test whether M16R is a second-site revertant, PCR analysis of its genome was carried out. This analysis showed that the 3' end of the M16R ICP27 gene retains the original dinucleotide mutation of M16, which is marked by an *Xho*I site (Fig. 1B; data not shown). Therefore, M16R is a second-site revertant of M16.

The growth properties of M16R were tested in a viral plaque assay, with WT HSV-1 and M16 serving as controls (Table 1, experiment 1). Consistent with previous results (28), WT HSV-1 formed plaques efficiently on both Vero and V27 cells whereas M16 was unable to form plaques on Vero cells. M16R formed plaques efficiently in both Vero and V27 cells, similar to the WT virus. However, the M16R plaques were considerably smaller than WT plaques (Fig. 2A), suggesting that M16R does not replicate or spread as well as WT HSV-1 in Vero cells.

To further study M16R's growth properties, single-cycle viral yield assays were carried out. Vero or V27 cells were infected in duplicate with WT HSV-1, M16, or M16R at an MOI of 10, and the infections were allowed to proceed for 24 h. Viral yields were determined by plaque assay of the cell lysates on V27 cells (Fig. 2B). As expected, WT HSV-1 replicated

TABLE 1. Plaque formation ability of viral ICP27 mutants

Virus stock	Titer (PFU/ml) on:		Vero/V27 cell plaque formation efficiency ratio
	Vero cells	V27 cells	
Expt 1			
WT	4.5×10^9	4.2×10^9	1.1
M16	$<2.0 \times 10^4$	4.9×10^8	<0.000041
M16R	6.3×10^{8a}	9.5×10^8	0.66
Expt 2			
WT	4.1×10^8	2.7×10^8	1.5
d27-1	$<2.0 \times 10^3$	2.9×10^8	<0.000069
M16R	3.6×10^{8a}	5.0×10^8	0.72
M16exC	$<2.0 \times 10^{4b}$	2.1×10^8	<0.000095
exCd305	$<2.0 \times 10^{4b}$	2.1×10^8	<0.000095
n217d	$<2.0 \times 10^{3b}$	1.2×10^8	<0.000017
Expt 3			
M16R	8.6×10^{8a}	8.9×10^8	1.0
exCd305	$<2.0 \times 10^{4b}$	8.5×10^8	<0.000024
R1exCd305	9.9×10^{8a}	8.2×10^8	1.2
R2exCd305	$2.8 \times 10^{8c,d}$	6.3×10^{8d}	0.44

^a Plaques are small compared to WT plaques.^b Discrete plaques are not visible, but some small foci of infected cells can be seen.^c Plaques are minute compared to WT plaques.^d A fraction of the plaques display a syncytial morphology.

efficiently in either cell line whereas M16 could only replicate in V27 cells. The growth phenotype of M16R was distinct from that of the two other viruses. In Vero cells, its yield was ~100-fold higher than that of M16, consistent with its ability to form plaques in these cells. However, its yield was ~100-fold reduced compared to that of the WT. M16R replicated ~10-fold more efficiently in V27 cells than in Vero cells, indicating that expression of WT ICP27 can enhance its growth. Taken together, the data from the plaque formation and yield experiments demonstrate that M16R can grow productively in Vero cells, albeit not as efficiently as WT HSV-1.

Identification of a frameshift mutation in M16R. A second-site reversion mutation in the M16R genome could be either in

the ICP27 gene (intragenic) or outside of it (extragenic). To test whether M16R has an intragenic reversion, we cloned its ICP27 allele and tested it in a viral complementation assay for the ability to complement the growth of *d27-1*, a viral ICP27 null mutant (27, 29). To carry out the assay, Vero cells were transfected in duplicate with pUC19 or a pUC19 derivative bearing the WT, M16, or M16R ICP27 allele. One day after transfection, the cells were infected with *d27-1*. Progeny virus were harvested after an additional day, and titers were determined by plaque assay on V27 cells (Fig. 3A). Consistent with previous assays (28), the WT ICP27 plasmid dramatically enhanced the growth of *d27-1* ($>10^5$ -fold increase in viral titer). In contrast, the M16 allele was completely unable to support *d27-1* growth. The M16R ICP27 plasmid exhibited significant complementation activity, increasing the growth of *d27-1* by ~1,000-fold. Since the ICP27 gene is the only intact HSV-1 gene on the transfected plasmid, this experiment suggests that M16R contains an intragenic reversion. To confirm this, we sequenced the entire 2.4-kb viral insert of the M16R plasmid. Only two differences from the sequence of WT strain KOS1.1 (which was determined in parallel) were found. First, as expected from the PCR analysis, the M16R ICP27 allele retains the original M16 dinucleotide alteration at codon 488. Second, whereas the WT gene has a run of eight C residues in the coding strand at codons 215 to 217, the M16R gene has a run of nine C's (Fig. 4A). The insertion results in a frameshift mutation, such that the M16R ICP27 allele is expected to encode a 289-residue polypeptide consisting of the first 217 residues of ICP27 fused to 72 novel C-terminal residues derived from the +1 reading frame (Fig. 4B).

This result was quite surprising, as much previous work suggests that the C-terminal portion of ICP27 is required for viral growth. To see if M16R expresses the predicted 289-residue protein, we carried out an immunoblot analysis. Vero cells were mock infected or infected with WT, M16, or M16R. Total proteins were isolated at 4 hpi and subjected to immunoblotting using H1113, a monoclonal antibody which recognizes residues 109 to 137 of ICP27 (18). As can be seen in Fig. 4C, WT and M16 express intact ICP27 molecules of ~63 kDa whereas M16R expresses a truncated molecule of ~38 kDa, a

FIG. 2. Growth of M16R in Vero cells. (A) Monolayers of Vero cells were infected with serial dilutions of WT HSV-1 or M16R. After 4 days, photographs of individual plaques were taken. (B) Replicate cultures of approximately 3×10^6 Vero or V27 cells were infected in duplicate at an MOI of 10 with the viral strains indicated. At 24 hpi, the infections were terminated by freezing and infectious virus was released by three cycles of freeze-thawing. The amount of infectious virus in each lysate was determined by plaque assay of the lysate on V27 cells and divided by the number of infected cells to determine the yield per cell.

FIG. 3. Complementation of *d27-1* by cloned ICP27 alleles. Replicate cultures of Vero cells were transfected in duplicate with pUC19 or pUC19 derivatives bearing various ICP27 alleles. One day after transfection, the cultures were infected with the HSV-1 deletion mutant *d27-1* and incubated for a further day. Virus was released from the cultures by three cycles of freeze-thawing, and the titers of the resulting lysates were determined by plaque assay of the lysates on V27 cells.

size consistent with that expected for the 289-residue protein. Immunofluorescence analysis was also carried out to determine the intracellular localization of the M16R protein. Vero cells were mock infected or infected with WT HSV-1, M16, or M16R. At 4 hpi, the cells were fixed and processed for immunofluorescence using the H1113 antibody. The polypeptides expressed by all three viruses were predominantly nuclear but differed in intranuclear localization (Fig. 4D). WT ICP27 was distributed throughout the nucleus in a speckled pattern, whereas the M16 protein was localized more diffusely and was largely excluded from nucleoli. The M16R protein showed a distinct localization pattern in that it was distributed diffusely in the nucleus but was preferentially localized to nucleoli. In summary, the results of these analyses indicate that M16R

expresses a truncated ICP27 molecule which localizes to the nucleus in an altered pattern.

Ability of frameshifted ICP27 alleles to complement *d27-1*. As noted above, it was surprising that the frameshifted M16R ICP27 gene was able to enhance the growth of *d27-1* in the viral complementation assay. To investigate this phenomenon further, we carried out a series of experiments using the viral complementation assay. First, to confirm that the frameshift is the bona fide intragenic reversion, we used site-directed mutagenesis to engineer this mutation into a plasmid bearing the M16 mutation. This construct, which we designated pM16exC (Fig. 1D), should be identical to the original M16R allele. In addition, we introduced the frameshift mutation into an otherwise WT ICP27 allele, generating WTexC (Fig. 1E). When

FIG. 4. The M16R frameshift mutation leads to the expression of a truncated, nucleus-localized ICP27 molecule. (A) The frameshift mutation of M16R. The coding strand sequences of codons 214 to 218 are shown for both the WT and M16R ICP27 alleles. The WT gene has a run of eight C residues; the M16R gene has nine. This leads to alteration of the encoded protein at residue 218 as shown. (B) Amino acid sequence (in single-letter code) of the frameshifted portion of the M16R ICP27 protein (residues 218 to 289). (C) Immunoblot analysis of the M16R protein. Vero cells were mock infected or infected with the viruses indicated. At 4 hpi, total proteins were harvested and analyzed by Western blot analysis using monoclonal antibody H1113, which recognizes residues 109 to 137 of ICP27. The molecular masses of marker proteins are indicated at the right. (D) Immunofluorescence analysis of the M16R protein. Vero cells were mock infected or infected with the WT, M16, or M16R virus, as indicated. At 4 hpi, the cells were fixed, permeabilized, and processed for immunofluorescence using the H1113 antibody.

tested in the viral complementation assay, both the M16R and M16exC alleles complemented *d27-1* growth by ~1,000-fold (Fig. 3B). This confirms that the frameshift mutation is the only change required in the M16 allele to endow it with enhanced complementation activity. The WTexC allele also showed ~1,000-fold complementation, demonstrating that the M16 alteration at codon 488 is not required.

The above-described results are consistent with the hypothesis that the complementation activity is mediated by the 289-residue protein. However, given the large amount of data which suggest that the C-terminal portion of ICP27 is essential for growth, we wished to rule out the possibility that this part of the protein was being expressed by some mechanism and contributing to the observed complementation. For example, the C-terminal region might be expressed via translational frameshifting, mRNA splicing, or the utilization of an internal ICP27 gene promoter. To help exclude these possibilities, we engineered a downstream nonsense mutation into the frameshifted gene at codon 406. This allele was designated exCn406 (Fig. 1F). We also constructed a gene, designated WTn406, in which the stop codon was engineered into the otherwise WT gene (Fig. 1G). When tested in the viral complementation assay, the nonsense mutation completely inactivated the WT ICP27 gene, consistent with previous results (17, 31). However, it had little, if any, effect on the frameshifted allele, which again showed ~1,000-fold complementation (Fig. 3C). The results suggest that the C-terminal portion of the ICP27 gene is not involved in the complementation activity. To address this issue more definitively, we engineered a frameshifted gene in which nearly all of the C-terminal coding region (codons 306 to

512) is deleted. This allele was designated exCd305 (Fig. 1H). When tested in the complementation assay, the exCd305 gene complemented *d27-1* as efficiently as the comparable non-deletion-containing allele, WTexC (Fig. 3D). Therefore, the complementation activity of the frameshifted alleles is mediated by the 289-residue protein, with no contribution from the C-terminal portion of ICP27.

To see if the novel C-terminal residues of the 289-residue protein (Fig. 4B) play a role in complementation, we engineered an ICP27 allele which specifically lacks these residues. This was done by introducing a nonsense mutation into the ICP27 gene at codon 218, which is the site at which the novel C-terminal residues begin (Fig. 4A). The mutation was made in the context of the codon 306 to 512 deletion, to rule out any possible effect of the C-terminal portion of ICP27. The altered gene was designated n217d (Fig. 1I). When tested for complementation, n217d showed only minimal activity (~5-fold) whereas the exCd305 allele again showed nearly 1,000-fold complementation (Fig. 3E). These results indicate that the novel 72 C-terminal residues play a critical role in the function of the 289-residue protein.

One or more extragenic mutations are required for the viability of M16R. Based on the above findings, we hypothesized that the intragenic frameshift mutation is sufficient to explain M16R's viability in Vero cells. To test this, we engineered a recombinant virus, designated M16exC, in which the WT ICP27 gene was replaced with the M16exC ICP27 allele (Fig. 1D). The M16exC allele was engineered to be identical to that of M16R. Therefore, if the frameshift mutation is sufficient for the viability of M16R, then the M16exC virus should

FIG. 5. ICP27-related proteins expressed by mutant viruses. Vero cells were mock infected or infected with the viruses indicated. At 6 hpi, total proteins were harvested and analyzed by immunoblot analysis using monoclonal antibody H1113, which recognizes residues 109 to 137 of ICP27. The molecular masses of marker proteins are indicated at the right. (A) Analysis of M16exC, exCd305, and n217d. (B) Analysis of R1exCd305 and R2exCd305.

be viable also. Two other recombinant viruses, exCd305 and n217d, were additionally constructed. These possess the ICP27 alleles shown in Fig. 1H and 1I, respectively. The recombinant viruses were isolated and amplified in V27 cells to ensure that no selection would be applied for a functional ICP27 molecule. For each mutant, two genetically independent isolates were obtained. One isolate was arbitrarily designated the primary isolate, to be used for extensive further analysis. The other isolate was designated the secondary isolate (and given the suffix b), to be used to confirm key findings. Extensive PCR analysis of all six isolates confirmed that their ICP27 alleles had the expected genomic structures (not shown).

Experiments were performed to characterize the ICP27-related proteins expressed by the various mutants. Immunoblotting analysis was used to characterize the sizes of the polypeptides. Total protein samples were prepared from infected cells at 6 hpi and analyzed by immunoblotting using the H1113 antibody (Fig. 5A). As expected, both M16exC and exCd305 expressed a major ICP27-related polypeptide which comigrated with that of M16R. In contrast, n217d expressed two major proteins smaller in size, the larger of which was roughly consistent with the size expected for its allele. In this experiment, both WT and mutant samples showed multiple protein bands. We suspect that the faster-migrating species are derived from the slower-migrating species by proteolysis, as their abundance varied between experiments (for example, compare the M16R patterns in Fig. 4C and 5). To characterize the localization of the mutant polypeptides, immunofluorescence analysis was performed using the H1113 antibody. At 4 hpi, all three polypeptides showed the same pattern of localization as the M16R protein (shown in Fig. 4D), i.e., predominant nuclear localization with preferential accumulation in nucleoli (data not shown). In both the immunoblotting and immunofluorescence experiments, the steady-state levels of the truncated proteins expressed by M16R, M16exC, exCd305, and n217d all appeared approximately equal (Fig. 5A and data not shown).

Next, the growth properties of the engineered viruses was examined by plaque analysis (Table 1, experiment 2). M16exC, exCd305, and n217d all formed plaques efficiently on V27 cells but were unable to form readily discernible plaques on Vero cells. This was in sharp contrast to M16R, which efficiently formed plaques on Vero cells. The three new mutants did form some small foci of infected cells that could be seen under microscopic examination but only when relatively low dilutions of the viral stocks were plated. Representative exCd305 and

n217d foci are shown in Fig. 6C and D, respectively. The foci produced by n217d were slightly smaller than those made by M16exC and exCd305. Plaque formation analysis of the secondary isolates of all three viruses gave essentially identical results (data not shown).

Single-cycle viral yield assays were also carried out. Cultures of Vero cells were infected in duplicate at an MOI of 10 with WT HSV-1, d27-1, M16R, M16exC, exCd305, or n217d. As controls, each inoculum was also used to infect cultures of V27 cells. The cultures were harvested at 24 h, and the yields were determined by plaque assay of the infected cell lysates on V27 cells (Fig. 7A). In V27 cells, all infections were comparably productive, achieving yields of ~100 PFU/cell. In Vero cells, however, the yields differed dramatically. As expected, WT HSV-1 replicated efficiently, producing ~100 PFU/cell; M16R replicated modestly, producing ~1 PFU/cell; and d27-1 did not replicate at all, producing <0.001 PFU/cell. Consistent with the results of the plaque formation analysis, M16exC, exCd305 and n217d all replicated significantly less efficiently than M16R. The yield of M16exC was ~500-fold reduced from that of M16R, whereas the yield of exCd305 was ~100-fold reduced. The mutant n217d displayed the most severe growth impairment, being essentially indistinguishable from d27-1 in its inability to produce infectious progeny.

Together, the results of the plaque formation and yield analyses lead to two important insights. The first is based on the comparison of M16R and M16exC. Although M16exC was engineered to have the same ICP27 allele as M16R, it clearly is not viable in Vero cells. The simplest interpretation of this finding is that M16R possesses an extragenic mutation which is required for its viability in addition to the intragenic frameshift alteration. The second insight is based on a comparison of the growth phenotypes of exCd305 and n217d. Although neither virus is able to efficiently form plaques in Vero cells, exCd305 produces significantly higher yields than n217d. M16exC, which also expresses the 289-residue protein, also replicates significantly better than n217d. Thus, it appears that the 289-residue, but not the 217-residue, ICP27 protein is able to enhance viral replication in infected cells, although not to a level sufficient for viability. This finding is consistent with the results of the viral complementation assay (Fig. 3E), which also indicated that the C-terminal frameshift segment is critical to the function of the truncated protein.

Isolation of viable revertants of exCd305. The above-described results suggest that M16R harbors one or more extra-

FIG. 6. Morphology of mutant plaques. Vero cell monolayers were mock infected (A) or infected with serial dilutions of the following viruses: M16R (B), *exCd305* (C), *n217d* (D), R1exCd305 (E), and R2exCd305 (F). After 4 days, photographs of individual plaques or foci were taken.

genic mutations which are required, in addition to its intragenic frameshift mutation, for viability. The relative ease with which M16R was isolated suggests that the frequency of such extragenic mutations is high. To test this hypothesis, we attempted to select viable revertants of *exCd305*, which possesses the frameshift mutation in the context of the C-terminally deleted ICP27 gene. Four *exCd305* plaques, generated on V27 cells, were amplified into small stocks. These were then passaged several times in Vero cells. Using this protocol, two *exCd305* plaques eventually yielded cultures that showed HSV-1 CPE and tested positive in a PCR assay for the presence of the ICP27 gene with a C-terminally deletion. Viral isolates from these cultures were purified and designated R1exCd305 and R2exCd305.

Southern blotting was carried out to characterize the ICP27 alleles of the two new revertants (Fig. 8A). A blot of *Pst*I-*Sal*I-digested viral DNAs was hybridized with a probe specific for the N-terminal portion of the ICP27 gene (Fig. 8B). As expected, all viruses except *d27-1* showed a hybridizing 1.8-kb fragment. However, when a C terminus-specific probe was used (Fig. 8C), no hybridization was observed for either *exCd305* or its revertants whereas appropriate hybridizing bands were seen for the other viruses. Note that *d27-1* shows a hybridizing band of 4.3 kb, similar to the WT band, since its deletion removes the *Sal*I site in the ICP27 gene and fuses the shortened *Pst*I fragments together. From these data, we conclude that both R1exCd305 and R2exCd305 retain the C-terminal deletion of their parent.

Immunoblotting was used to characterize the ICP27-related polypeptides synthesized by the revertants (Fig. 5B). Both revertants expressed truncated ICP27-related proteins that comigrated with those of *exCd305* and M16R and that were expressed at similar levels. In addition, immunofluorescence analysis showed that the ICP27 proteins of R1exCd305 and R2exCd305 localized similarly to that of their parent; i.e., they exhibited nuclear localization with preferential accumulation in nucleoli (data not shown).

Next, the growth phenotypes of R1exCd305 and R2exCd305 were analyzed by plaque assay. Both mutants differed dramatically from their parent in the ability to form plaques on Vero cells (Table 1, experiment 3). Whereas *exCd305* had a Vero/V27 cell plaque formation ratio of <0.000024 , R1exCd305 and R2exCd305 had ratios that approximated 1, similar to that of M16R. This indicates that the two mutants are viable in Vero cells. The R1exCd305 plaques on Vero cells (Fig. 6E) were approximately the same size as those made by M16R (Fig. 6B), whereas the R2exCd305 plaques were somewhat smaller (Fig. 6F). A minor fraction of the R2exCd305 plaques displayed a syncytial morphology; the significance of this observation is unknown.

To further characterize the growth abilities of R1exCd305 and R2exCd305, single-cycle growth assays were carried out (Fig. 7B). The WT, *d27-1*, M16R, and *exCd305* viruses were included for comparison. As expected, all grew efficiently in V27 cells. In Vero cells, R1exCd305 and R2exCd305 produced approximately 100- and 10-fold more progeny than their parent, respectively. The growth of R1exCd305 was comparable to that of M16R, consistent with the fact the two viruses make plaques similar in size on Vero cells. R2exCd305, on the other hand, grew somewhat less efficiently than either R1exCd305 or M16R, consistent with its smaller plaque size. Together, the data from the plaque formation and yield experiments demonstrate that both *exCd305* revertants have acquired mutations which allow them to grow more efficiently and form plaques in Vero cells.

The Southern blot analysis indicates that R1exCd305 and R2exCd305 retain the ICP27 gene C-terminal deletion, but it is possible that they have mutations elsewhere in their ICP27 coding sequences. To exclude this possibility, the ICP27 coding regions of both mutants were cloned and sequenced in their entirety. No mutations, relative to the *exCd305* allele, were present in either revertant. Therefore, we conclude that the reversion mutations of both mutants are extragenic. We also sequenced 413 bp upstream and 471 bp downstream of each

coding region. No changes were found for R1exCd305, but R2exCd305 possessed a single nucleotide mutation 214 bp upstream of the ICP27 gene transcriptional start site (a C-to-A alteration). The significance of this difference is currently under investigation.

Viral DNA replication in mutant-infected cells. Viral DNA replication is a central event in viral lytic infection, amplifying viral genomes for later packaging into progeny particles. In addition, the process of DNA replication confers a *cis*-acting change in the viral template which is associated with a switch from DE to L gene expression (13). ICP27 has an important role in activating viral DNA replication, as it stimulates the levels of replicated DNA by 5- to 10-fold (14, 27, 39). As an initial step in understanding how the intra- and extragenic mutations of M16R and the other relevant mutants affect the outcome of infection, we examined the ability of various mutants to replicate their DNA. In the first experiment, Vero cells were mock or HSV-1 infected and total DNA was purified from the cultures at either 1 or 12 hpi. Equal amounts of the DNA were double digested with *Pst*I and *Sa*I and subjected to Southern blot analysis using an HSV-1-specific probe (Fig. 9A). To quantitate the degree of DNA replication by each mutant, the hybridization signal at 12 hpi was divided by the signal at 1 hpi to determine the fold DNA amplification (Fig. 9B). The results indicated that WT HSV-1 replicated its DNA to the greatest degree, followed in order by R2exCd305, M16R, exCd305, and M16exC, which all showed lower but significant DNA replication. In contrast, R1exCd305 showed very low (and possibly insignificant) DNA amplification whereas d27-1 and n217d exhibited essentially no DNA replication.

We hypothesized that some of the mutants have delayed DNA replication relative to that of the WT virus. Therefore, we carried out a second experiment in which DNA was prepared at 16 rather than 12 hpi. The results are shown in Fig. 9C. As before, WT HSV-1 replicated its DNA the most efficiently whereas d27-1 was unable to replicate its DNA to any significant extent. M16R, M16exC, exCd305, R1exCd305, R2exCd305, and n217d all replicated their DNAs to intermediate levels. Thus, R1exCd305, which was borderline for DNA replication in the first experiment, clearly is able to replicate more DNA than d27-1. The virus that differed the most between the two experiments was n217d. In this experiment, it replicated a significant amount of viral DNA whereas it was negative for DNA replication at 12 hpi.

The above-described results suggest that n217d is competent for DNA replication but delayed in the kinetics of DNA accumulation compared to the other mutants. To test this hypothesis, we performed a time course analysis of DNA replication comparing n217d, the WT, d27-1, and exCd305 at 8, 12, 16, and 24 hpi. The results, shown in Fig. 9D, confirm that n217d is able to replicate DNA more efficiently than d27-1, by about threefold at 16 to 24 hpi. Of note was the fact that exCd305 replicated its DNA comparably to the WT virus in this experiment. However, we note that since our procedure only measures cell-associated DNA, it may underestimate the amount of DNA at late times for the WT virus, since a significant amount of viral progeny may be secreted into the medium.

Three conclusions can be drawn from the DNA replication experiments. The first is based on the observation that all viruses expressing the 289-residue frameshifted protein, including nonviable mutants such as M16exC, are able to replicate appreciably more viral DNA than d27-1. Therefore, we conclude that the 289-residue protein is able to significantly enhance viral DNA replication. Second, there is no clear correlation between the ability of the various frameshift mutants to replicate DNA and their viability in Vero cells. It follows,

FIG. 7. Replication ability of mutant viruses. Replicate cultures of Vero or V27 cells were infected in duplicate at an MOI of 10 with the viral strains indicated. At 24 hpi, the infections were terminated and infectious virus was released by three cycles of freeze-thawing. The amount of infectious virus in each lysate was determined by plaque assay on V27 cells. (A) Analysis of M16exC, exCd305, and n217d. (B) Analysis of R1exCd305 and R2exCd305.

therefore, that the extragenic mutations of the revertants, which are required for viability, must affect an essential function other than DNA replication. Third, n217d replicates slightly more viral DNA than an ICP27 null mutant. Based on this, we conclude that the N-terminal 217 residues of ICP27 have an inherent but weak ability to stimulate viral DNA replication.

DISCUSSION

HSV-1 mutants that replicate in the absence of the C-terminal half of ICP27. In this paper, we describe the isolation of three HSV-1 mutants (M16R, R1exCd305, and R2exCd305) which grow productively in cultured cells despite the fact that they do not express an intact ICP27 molecule. Instead, due to a frameshift mutation, they produce an aberrant and highly truncated form of ICP27 that completely lacks its C-terminal half. In the case of M16R, we cannot exclude the possibility that the C-terminal portion of ICP27 is expressed via some mechanism such as mRNA splicing or utilization of an alternate promoter. We do not have an antibody which recognizes the C-terminal part of the molecule, so it is difficult to exclude this possibility. However, in R1exCd305 and R2exCd305, the coding sequences for the C-terminal segment have been completely deleted. Therefore, we can conclude that the C-terminal half of ICP27 is not absolutely required for productive

FIG. 8. Southern blot analysis of *exCd305* revertants. (A) Schematic diagram of expected Southern blot results. The arrow at the top indicates the ICP27 coding region, and the lines denote relevant restriction fragments in the ICP27 region of the WT and mutant HSV-1 genomes. (B and C) Viral genomic DNAs were digested with *Pst*I and *Sal*I and hybridized with a radioactive probe specific for the N-terminal (B) or C-terminal (C) region of the ICP27 gene (denoted by gray bars in panel A). The positions of DNA size standards are shown at the right of each autoradiogram.

growth of HSV-1 in cultured cells, although as discussed below, it appears that compensatory mutations are required for the viability of these unusual mutants.

It is accurate to describe M16R, R1*exCd305*, and R2*exCd305* as viable for at least two reasons. First, they efficiently form plaques on Vero cells, as demonstrated by the fact that mutant stocks give nearly equal titers on Vero and V27 cells. Second, when seeded at a low MOI on Vero cells, they efficiently spread across the monolayer to infect and destroy all of the cells. Of course, viability is a viral phenotype that is highly dependent on the host cells used. Vero cells are particularly good hosts for HSV-1, possibly due to their inability to produce type I interferons (6, 7). It will be interesting to see whether the revertants grow productively in other types of cultured cells or in animal models of HSV-1 infection.

The finding that the C-terminal half of ICP27 is dispensable for viral growth, even in the presence of compensating mutations, was quite unexpected because a large amount of previous data implies that this part of the polypeptide is critical for its regulatory functions. For example, numerous mutations in the C-terminal half of ICP27 destroy the protein's ability to positively or negatively regulate cotransfected genes in transient-transfection assays (4, 9, 17, 31). Based on the results of such experiments, Sandri-Goldin and colleagues proposed several years ago that the C-terminal 249 and 62 residues of ICP27 comprise its "activation" and "repression" domains, respectively (9). Mutations in the C-terminal repressor domain destroy ICP27's abilities to induce the nuclear redistribution of cellular splicing factors (35), inhibit pre-mRNA splicing (8, 36), and shuttle between the nucleus and cytoplasm (19). Moreover, there is strong genetic evidence for the importance of the C-terminal portion of ICP27, as many mutations in the

C-terminal half of the ICP27 gene are lethal when introduced into recombinant viruses (17, 27, 28).

Despite the fact that the revertants are viable, they are attenuated in their growth compared to WT HSV-1. The mutant plaques are small, and their yields in single-cycle growth assays are reduced ~100-fold compared to that of the WT. This growth defect can be attributed to a defect in ICP27, since expression of WT ICP27 from the stably transfected gene of V27 cells significantly complements the growth of the mutants in plaque formation and yield assays (Table 1; Fig. 2B and 7). Therefore, although our studies indicate that the C-terminal part of ICP27 is dispensable in some circumstances, they also demonstrate that it plays a significant role in viral growth.

The truncated, frameshifted ICP27 molecule significantly enhances viral growth. The frameshift mutation in M16R and the other mutants in this study results in the expression of a truncated 289-residue polypeptide consisting of the N-terminal 217 residues of ICP27 fused to 72 novel C-terminal residues. Our results indicate that this protein exhibits a significant regulatory function in infected cells. First, it can enhance the growth of the viral ICP27 null mutant *d27-1* by ~1,000-fold in a transfection-based complementation assay. Second, the engineered mutants M16*exC* and *exCd305*, which express the 289-residue protein, exhibit significantly higher viral yields in Vero cells than does *d27-1*. It was somewhat surprising that the M16R ICP27 allele was able to complement *d27-1* growth by ~1,000-fold in the transfection assay (Fig. 3) but only led to an ~10- to 100-fold increase in viral yield relative to *d27-1* when introduced into a recombinant virus (Fig. 7A). We speculate that artificially high expression of the mutant protein in transfected cells enhances its ability to stimulate the production of viral progeny.

FIG. 9. Viral DNA synthesis in mutant-infected Vero cells. Replicate cultures of Vero cells were mock infected or infected with WT HSV-1 or ICP27 mutants at an MOI of 10. At 1 hpi and various times thereafter, total DNA was isolated from the cultures. Five micrograms of each DNA sample were double digested with *Pst*I plus *Sal*I, and the digests were subjected to Southern blot analysis using a probe specific for the UL55 open reading frame. This probe detects a 4.15-kb fragment in the WT HSV-1, M16, M16R, and M16exC genomes. In *d27-1*, due to the deletion in the ICP27 (UL54) gene which removes a *Sal*I site, the probe detects a 4.3-kb fragment. In *exCd305*, *n217d*, *R1exCd305*, and *R2exCd305*, due to the deletion of the C terminus-encoding region, the probe detects a 3.5-kb fragment. (A) Viral DNA accumulation at 12 hpi. An autoradiograph of the Southern blot is shown, and the positions of DNA size standards are indicated to the right. (B) Quantitation of fold DNA amplification at 12 hpi. The filter used for panel A was analyzed by phosphorimager analysis. For each infecting virus, the amount of hybridization signal at 12 hpi was divided by the 1-hpi signal to derive the fold DNA accumulation. (C) Quantitation of fold DNA amplification at 16 hpi. (D) Quantitation of fold DNA amplification in a time course (8, 12, 16, and 24 hpi).

What function does the 289-residue protein perform in infected cells? Our analysis of viral DNA replication provides a likely answer. Although there was some variability in the overall levels of viral DNA replication in our experiments, we consistently found that all of the mutants expressing the 289-residue protein, including the nonviable mutants, replicated significantly more viral DNA than did *d27-1* (Fig. 9). Thus, the 289-residue protein is able to mediate the DNA replication enhancement function of ICP27. Although the source of the variability in the DNA replication experiments is unknown, it may relate to the fact that fold DNA replication in our assay is determined by dividing a relatively large number (the units of accumulated DNA at a late time point) by a much smaller number (units of DNA at 1 hpi) which, in some cases, is close to the background signal and thus difficult to measure accurately.

The finding that an N-terminal form of ICP27 can stimulate viral DNA replication is consistent with some of our past results. We previously found that an N-terminal ICP27 mutant, *d1-2*, is deficient for viral DNA synthesis but shows only modestly reduced expression of several L genes (29). Conversely, we showed that several C-terminal ICP27 mutants have the opposite phenotype; i.e., they are proficient at viral DNA replication but are unable to efficiently express L genes (27, 28).

Based on these observations, we previously proposed that ICP27 mediates two separable functions: (i) an N-terminal dependent activity which stimulates viral DNA synthesis and (ii) a C-terminal dependent activity which stimulates L gene expression independently of the stimulatory effect of viral DNA replication itself (27–29). The results of our present study are consistent with this model and further implicate the N-terminal segment of ICP27 in the DNA replication function.

An unexpected and still puzzling finding is that the novel 72 residues at the C terminus of the 289-residue polypeptide, derived from the +1 reading frame, play a critical role in the function of the truncated protein. One possibility is that these residues carry out a highly specific function that endows the N-terminal fragment of ICP27 with a novel activity. However, a more likely explanation is that these residues rescue or stabilize an inherent but weak activity encoded in ICP27's N-terminal 217 residues. Evidence for the latter hypothesis comes from analysis of the viral mutant *n217d*, which expresses only the N-terminal 217 residues. Although this mutant does not grow in Vero cells, it does accumulate slightly more viral DNA than the null mutant, suggesting that the N-terminal 217-residue fragment has an inherent but weak ability to stimulate viral DNA replication.

How might the 72 novel C-terminal residues enhance the

N-terminal activity? The sequence of the frameshift segment may provide a clue. Although BLAST searches fail to reveal similar sequences in current protein databases, it is striking that the frameshift sequence is quite arginine and glycine rich (Fig. 4B). This makes it very similar in composition to ICP27's known RNA-binding domain, which consists of a short 15-residue stretch with similarity to RGG box-type motifs found in other proteins (20). This sequence maps to residues 138 to 152 and is thus present in both the 217- and 289-residue proteins. It is possible that the frameshift segment confers an additional RNA-binding activity or augments RNA binding by the RGG box. There are several other possible mechanisms by which the frameshift sequence might enhance the function of the N-terminal fragment. For example, it might increase the stability of the truncated polypeptide or allow it to localize more efficiently to the cellular compartment(s) where it acts. These explanations appear unlikely, however, since both the 217- and 289-residue fragments are expressed at comparable levels in infected cells and exhibit similar nuclear localization patterns characterized by preferential accumulation in nucleoli. Lastly, it is possible that the frameshift segment enhances protein multimerization. This hypothesis is consistent with recent work by Zhi et al. which demonstrated that ICP27 multimerizes *in vivo* and that the C-terminal half is critical for this ability (42). In this scenario, the frameshift segment could allow for the self-association of ICP27, thereby bypassing the need for the multimerization function normally supplied by the C-terminal half.

Extragenic mutations are required for the viability of viruses expressing frameshifted ICP27. To test whether the intragenic frameshift mutation is the only alteration required for M16R's viability, we re-engineered the M16R ICP27 allele and introduced it into a new virus, M16exC, in place of the WT gene. However, M16exC is not viable in Vero cells, nor is another mutant, exCd305, which was also engineered to express the frameshifted gene. The simplest interpretation of these results is that M16R possesses one or more extragenic mutations which are required for viability in addition to the frameshift mutation. Consistent with this, we were able to readily select two viable derivatives of exCd305, designated R1exCd305 and R2exCd305. Sequencing analysis revealed that these revertants have no unexpected alterations in their ICP27 genes. Therefore, their viable phenotypes must result from extragenic mutations. It is noteworthy that the growth phenotypes of the two revertants are distinct, suggesting that their extragenic reversion mutations are also distinct.

What is the source of the extragenic mutation(s) in M16R? In this regard, it is instructive to contrast the isolation of M16R, which replicates in Vero cells, with that of M16exC, which does not. In the case of M16R, multiple passages and plaque purifications were performed in Vero cells, as was preparation of the initial low-titer stock. In the case of M16exC, however, all genetic manipulations were carried out in V27 cells to minimize any potential selective pressure for additional mutations which could enhance growth. Based on these considerations, we hypothesize that the extragenic mutation(s) of M16R was acquired during its initial, prolonged selection in Vero cells, possibly after the acquisition of the frameshift mutation. This model is consistent with the proven sequential acquisition of the frameshift and extragenic mutations in the genomes of R1exCd305 and R2exCd305.

Taken together, our results indicate that at least two mutations are required to allow HSV-1 to replicate in Vero cells in the absence of its C-terminal domain. The first is the intragenic +1 frameshift mutation, which expands by one nucleotide a homopolymeric run of C residues in the ICP27 coding strand at

codons 215 to 217. Sasadeusz et al. have shown that expansion and contraction of such homopolymeric sequences appear to be common mutational events in HSV (37). The frameshift mutation results in expression of the 289-residue ICP27 protein, which is able to enhance viral DNA replication. We propose that the second mutation is an alteration outside of the ICP27 gene; it is feasible that multiple extragenic mutations are required. The effect of the extragenic change(s) is unknown. However, since mutants possessing only the intragenic change are able to undergo significant DNA replication, it seems likely that the extragenic alterations affect a step in viral growth after the onset of DNA synthesis. Given the known role of ICP27 in L gene expression, we speculate that this step is the activation of L genes. Our preliminary analysis of protein synthesis patterns of the various mutants supports this hypothesis, in that the viable revertants appear to express a wider range of viral proteins than do M16exC and exCd305 (S. Bunnell and S. Rice, unpublished data, 2000).

To understand the nature and effect of the extragenic mutations, it is necessary to identify these alterations at the nucleotide level. We have attempted to use a marker rescue approach to map M16R's extragenic mutation(s) by asking if restriction fragments derived from M16R can recombine into the M16exC genome and confer viability. However, we have observed that M16exC has a relatively high rate of spontaneous reversion, a fact that has hampered our mapping attempts. In addition, mapping of the extragenic mutation(s) of the revertants will be difficult if multiple extragenic alterations contribute to viability.

Does ICP27 mediate distinct N- and C-terminal functions?

Based on these and our past results, we propose that ICP27 carries out (at least) two distinct regulatory functions in infected cells, one of which is mediated by an N-terminal region and the other of which is dependent upon C-terminal sequences. We propose that the N-terminal activity is essential and acts to stimulate viral DNA replication, likely indirectly by transactivating DE genes that encode DNA replication proteins (15, 39). Although the N-terminal function can work in the complete absence of C-terminal sequences (at least in the context of the frameshift mutation), we do not know whether the C-terminal function depends upon N-terminal regions such as the RGG box (20) or nuclear export signal (34). This model can help explain why the ICP27 homologues of varicella-zoster virus and human cytomegalovirus, which bear homology to the C-terminal, but not the N-terminal, part of ICP27, are unable to complement the growth of HSV-1 ICP27 mutants (21, 41). That is, these molecules may be unable to perform the essential N-terminal function. One finding that our model does not readily explain, however, is why many ICP27 C-terminal mutations (such as M16) are so highly lethal. One possibility is that ICP27 sequences C terminal to residue 217 can inhibit the function of the N-terminal segment, possibly via direct physical interaction. This inhibition would be relieved by truncation of the protein by the frameshift mutation. More work is required to test this hypothesis.

In summary, we report the isolation of novel HSV-1 mutants which replicate in cultured cells despite the fact that they do not express the evolutionarily conserved C-terminal half of ICP27. We also show that compensatory mutations are required for the viability of these mutants. This study provides further evidence that ICP27 carries out multiple independent functions during viral lytic infection and indicates that ICP27's ability to enhance DNA replication is associated with the N-terminal half of the molecule. Further characterization of these unusual mutants is likely to illuminate the structure, function, and evolution of HSV-1 ICP27.

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