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Shuttling of the Herpes Simplex Virus Type 1 Regulatory Protein ICP27 between the Nucleus and Cytoplasm Mediates the Expression of Late Proteins

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The herpes simplex virus type 1 (HSV-1) immediate-early protein ICP27 is required posttranscriptionally for the expression of HSV-1 late genes during a productive infection. ICP27 also inhibits host cell pre-mRNA splicing, effectively shutting off host cell protein synthesis. Here we describe intragenic suppressors of LG4, a virus with a conditional lethal mutation in the gene encoding ICP27. At the restrictive temperature, *ts*ICP27 from LG4 fails to inhibit host cell pre-mRNA splicing and to activate the expression of HSV-1 late-gene products. Although the suppressors of LG4 restore virus growth, they still fail to inhibit host cell pre-mRNA splicing. Thus, the role of ICP27 in the synthesis of late proteins is independent of host shutoff. In HSV-1-infected cells, ICP27 shuttles between the nucleus and the cytoplasm. Shuttling of ICP27 occurs only at late times during infection. In transfected cells, ICP27 shuttling was dependent on coexpression of RNA from a late HSV-1 gene. While shuttling does not occur in cells infected with LG4 at 39.5°C, the suppressors of LG4 restore shuttling. Temperature shift experiments correlate the defect in shuttling with the temperature-sensitive phenotype of LG4. These data provide a correlation between shuttling of ICP27 and the expression of HSV-1 late-gene products. We propose that ICP27 regulates late-gene protein synthesis by facilitating the export of late RNAs.

During a productive infection, herpes simplex virus type 1 (HSV-1) gene expression proceeds in a tightly regulated cascade. Based on their temporal expression during a productive infection, the genes of HSV-1 are classified into three kinetic classes, immediate-early (α), early (β), and late (γ) (14, 15, 41). The immediate-early genes (α27, α4, α0, α22, α47, and αX [OrfP] [18, 19, 55]) are the first to be transcribed, and they are defined by their expression in the absence of de novo protein synthesis (1, 41). The α gene products, infected-cell protein 4 (ICP4), ICP0, ICP27, and ICP22, cooperatively regulate the expression of all kinetic classes of virus genes (4, 5, 8, 9, 22, 29, 30, 35, 39, 41, 42).

The essential immediate-early protein ICP27 is a multifunctional protein that is required for the expression of β and γ genes. How ICP27 activates the expression of β genes is unknown. However, ICP27 activates the expression of HSV-1 γ genes at the posttranscriptional level (22, 23, 36–39, 47, 50). The role of ICP27 in the activation of β genes is separable from its role in the activation of γ genes (43, 47, 52). Another function of ICP27 is to shut off host cell protein synthesis by inhibiting the splicing of host pre-mRNAs (10, 12, 32, 33, 44, 45, 47, 50). This function may not directly affect the expression of the majority of virus genes, since they do not contain introns. ICP27 contains an RGG box, which is required for RNA binding and for the role of ICP27 in the expression of γ gene products (13, 24). Although several virus RNAs are bound in vitro by ICP27, the RNA target site in vivo is unknown (2, 16, 25).

To further investigate the role of ICP27 in the expression of late-gene products, we studied LG4, a temperature-sensitive (*ts*) mutant of HSV-1. The defect in LG4 results from a single amino acid substitution, R480H, in ICP27 (11, 46). In cells infected with LG4 at the restrictive temperature, host cell protein synthesis is not shut off and late proteins are not detected (10–12, 47, 50). However, this virus is not defective in activation of β gene expression, as evidenced by its ability to replicate virus DNA. Intragenic suppressors of the LG4 mutation were isolated to study the functions of ICP27 that are disrupted by this mutation.

Analyses of the intragenic suppressors of LG4 separate the role of ICP27 in the shutoff of host cell protein synthesis from its role in the production of γ gene proteins. They also reveal that ICP27 shuttles between the nucleus and the cytoplasm at late times postinfection. Finally, a requirement for virus RNA in shuttling of ICP27 is demonstrated.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, N.Y.) containing 5% bovine calf serum (HyClone Laboratories, Inc., Logan, Utah) and supplemented with 100 U of penicillin and 100 μg of streptomycin (Gibco BRL) per ml. The ICP27-complementing cell line 2-2 is a derivative of Vero cells which expresses ICP27 under its own promoter (48). These cells were maintained in DMEM supplemented with 500 μg of G418 (Geneticin; Gibco BRL) per ml.

The strain of wild-type HSV-1 used in this study was KOS1.1A. The transplacement vector vBSΔ27 contains a *lacZ* gene fragment in place of the sequences encoding ICP27. It was generated by homologous recombination with linearized pBSp27ZX. The growth properties of LG4 have been described previously (11, 46). Revertants of LG4, 2-3, 3-3, 4-3, and 5-3 were isolated after three sequential plaque purifications at the restrictive temperature of 39.5°C. The viruses vBSΔ27R, vBSLG4, vBS2-3, vBS3-3, vBS4-3, and vBS5-3 were generated from vBSΔ27 by marker rescue with a *Bam*HI-linearized fragment containing the α27 allele from plasmids pBS27, pBSLG4, pBS2-3, pBS3-3, pBS4-3, and pBS5-3, respectively.

Plasmids. pBS27 was generated by cloning the α27 locus from KOS1.1A as a *Bam*HI-*Sac*I fragment into pIBI31. Plasmids pBSLG4, pBS2-3, pBS3-3, pBS4-3, and pBS5-3 were cloned from virus DNAs of LG4 and the revertants 2-3, 3-3,

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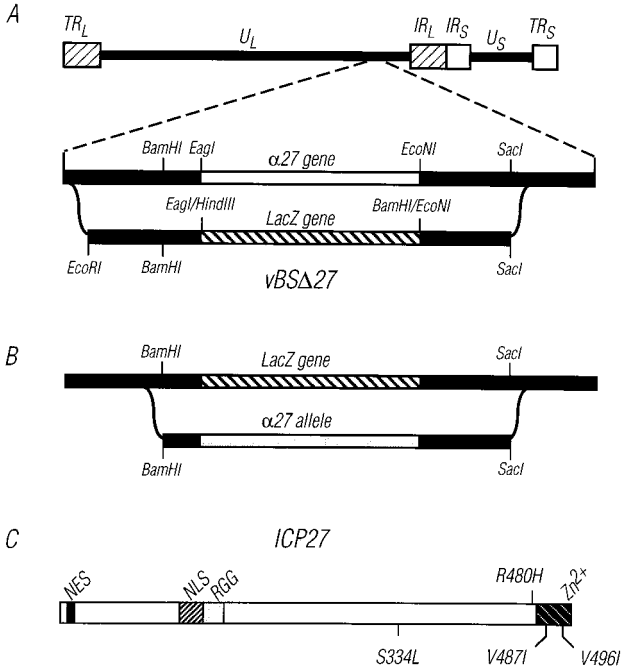

FIG. 1. Schematic diagram of the HSV-1 genome. (A) Schematic representation of the HSV-1 genome, the location of the α27 gene, and the boundaries of the transplacement virus. (B) The recombination strategy, including the restriction sites, used to isolate the various alleles of α27 and to generate recombinant viruses carrying these alleles. (C) Locations of defined domains of ICP27 and the sites of the LG4 mutation and its suppressors. The domains include the putative NES, the NLS, an RGG box that has been implicated in binding RNA, and its carboxy-terminal zinc finger. The *ts* mutation (R480H) from LG4 is shown above the line, and the locations and codon changes of the suppressors are shown below the line.

4-3, and 5-3, respectively, as *Bam*HI-*Sac*I fragments. The nucleotide sequence of the α27 open reading frame (ORF) in these plasmids was determined by cycle sequencing with the Ampli-Cycle sequencing kit (Perkin-Elmer, Branchburg, N.J.) and a series of primers complementary to the α27s ORF.

pBSp27ZX was constructed as follows. The *Eag*I-*Eco*NI fragment of α27 was removed from pBS27. The ends were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I, and an 8-bp *Bgl*II linker was inserted to create pBSΔ27. *lacZ* coding sequences were isolated from pCH110 (Pharmacia, Piscataway, N.J.) by digestion with *Hin*dIII, and an 8-bp *Bgl*II adapter was ligated to the filled ends of the linearized plasmid. The resulting DNA was digested with *Bam*HI and *Bgl*II to release a fragment containing the *lacZ* coding sequences. This fragment was inserted into the *Bgl*II site of pBSΔ27 to create pBSΔ27Z. The α27/*lacZ* cassette from pBSΔ27Z was removed as a *Bam*HI-*Eco*RI fragment and inserted into pAT153 to create pBSp27Z. pBSp27ZX was derived from pBSp27Z by insertion of a 3-kb *Eco*RI (filled)-*Bam*HI fragment containing the 5′ end of the HSV-1 α27 promoter between an *Eco*NI (filled) site and the *Bam*HI site (Fig. 1A).

The construct pRAB14 expresses α-*trans*-inducing factor (α-Tif) (UL48) under the α4 promoter (1). pBS3term was constructed by inserting the 5′CTAGT CTAGACTAG3′ (New England Biolabs, Beverly, Mass.) linker into the *Asp*718 site of pRAB14 after end-filling the site. Insertion of this linker places a translational terminator in all reading frames at +542 relative to the ATG. Thus, the resulting α-Tif peptide would be truncated at amino acid 181. pBSΔSac was constructed by digesting pRAB14 with *Sac*I and religated to remove an internal fragment containing the majority of the coding region. Deletion of the *Sac*I fragment leaves the coding sequence from amino acids 423 to 491 (end) intact, with a potential initiation codon at amino acid 470, and also spares the 3′ untranslated region.

Protein synthesis assays. Vero cells (10^6) were seeded in 60-mm tissue culture dishes and infected with the indicated viruses in absorption medium (DMEM supplemented with 1% bovine calf serum) at 10 PFU per cell for 1 h at 39.5°C. At the indicated time postinfection, the cells were washed three times with Met-free DMEM and incubated for 30 min in 200 μl of Met-free DMEM containing 50 μCi of Tran^{35}S Label (1,214 Ci/mmol; ICN Pharmaceuticals Inc., Costa Mesa, Calif.). The cells were then washed with ice-cold phosphate-buffered saline (PBS), resuspended in 300 μl of 1.5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and boiled for 5 min.

TABLE 1. Titers of wild-type HSV-1 and an ICP27 transplacement vector

Virus	Titer[a] on:	
	Vero cells	2-2 cells
KOS1.1A	4×10^9	4×10^9
vBSΔ27	<1	2×10^9
vBSΔ27R	1×10^9	1×10^9

[a]PFU per milliliter.

The proteins were analyzed by SDS-PAGE on 7.5% gels. The gels were fixed and treated with Entensify (NEN-DuPont, Boston, Mass.) and exposed to X-ray film.

RNA extraction and Northern blot analysis. Total cellular RNAs were prepared from mock-infected or HSV-1-infected Vero cells by a modification of the method of Chomczynski and Sacchi (3) with the commercially available TRIZOL reagent (Gibco BRL). Northern blot analysis was performed by electrophoresis of RNA in 1.5% agarose–6% formaldehyde gels by the method of Lehrach et al. (20). The gel was blotted by capillary action to GeneScreen Plus (NEN-Dupont) membranes with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The blot was hybridized with a ^{32}P-labeled DNA probe prepared by random priming a gel-isolated DNA fragment, containing a human β-actin gene, as specified by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Virus growth assays. Vero cells (10^6) were seeded in 35-mm dishes and infected the next day at a multiplicity of infection (MOI) of 0.1. The infections were allowed to proceed at either 39.5 or 32°C for the time indicated and were halted by freezing the cells at −80°C. The infected cells were subjected to five cycles of freezing and thawing, and the virus yields were obtained by titer determination on Vero cells at 32°C. Growth curves represent the mean of three independent infections, each titrated in duplicate.

Immunofluorescence. (i) Transfected ICP27. Transfections were performed by a modification of the calcium phosphate precipitation method (54). Briefly, 5×10^4 Vero cells were seeded in a 35-mm plate containing a glass coverslip; the next day, they were transfected with 10 μg of total plasmid DNA. The transfection mixtures were left on the cells for 16 h, and the medium was replaced after the cells were shocked with 15% glycerol in PBS for 60 s. Before being fixed, some samples were treated for 1 h with 4 μg of actinomycin D (Gibco BRL) per ml. Some samples were also treated with 100 μg of cycloheximide (Sigma, St. Louis, Mo.) per ml. The cell monolayers were rinsed with PBS, fixed with 3.7% formaldehyde in PBS for 30 min, permeabilized with 100% acetone at −20°C for 10 min, and stored at 4°C in PBS. ICP27 was detected with rabbit polyclonal antibody CLU38 (31) diluted 1:200 in PBS. The primary antibody was visualized with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Kirkegaard & Perry, Gaithersburg, Md.).

(ii) Infections. Vero cells (5×10^4), seeded on coverslips in 35-mm plates, were infected at an MOI of 0.01. The cells were fixed, and permeabilized at 6, 9, and 12 h postinfection as described above. ICP27 was detected as described above. ICP4 was detected with monoclonal antibody H1114, diluted 1:200 in PBS (Goodwin Institute, Plantation, Fla.). FITC- or tetramethyl-rhodamine isothiocyanate-conjugated secondary antibodies, i.e., goat anti-rabbit IgG and goat anti-mouse IgG (Kirkegaard & Perry), were used to determine the intracellular localization of ICP27 and ICP4, respectively.

(iii) Microscopy. Preparations were viewed in a Leitz Dialux microscope with optical systems for the selective visualization of fluorescein or rhodamine. Representative fields of cells were photographed with Ektachrome P1600 film (Eastman Kodak, Rochester, N.Y.) and a Nikon UFX-DXII photographic system.

TABLE 2. Virus titers at the permissive and restrictive temperatures

Virus	Titer[a] at:	
	39.5°C	32°C
KOS1.1A	4×10^9	4×10^9
LG4	4×10^5	1×10^9
vBSLG4	<1	1×10^9
vBS2-3	2×10^8	4×10^8
vBS3-3	5×10^8	5×10^8
vBS4-3	2×10^8	2×10^8
vBS5-3	5×10^7	5×10^7

[a]PFU per milliliter.

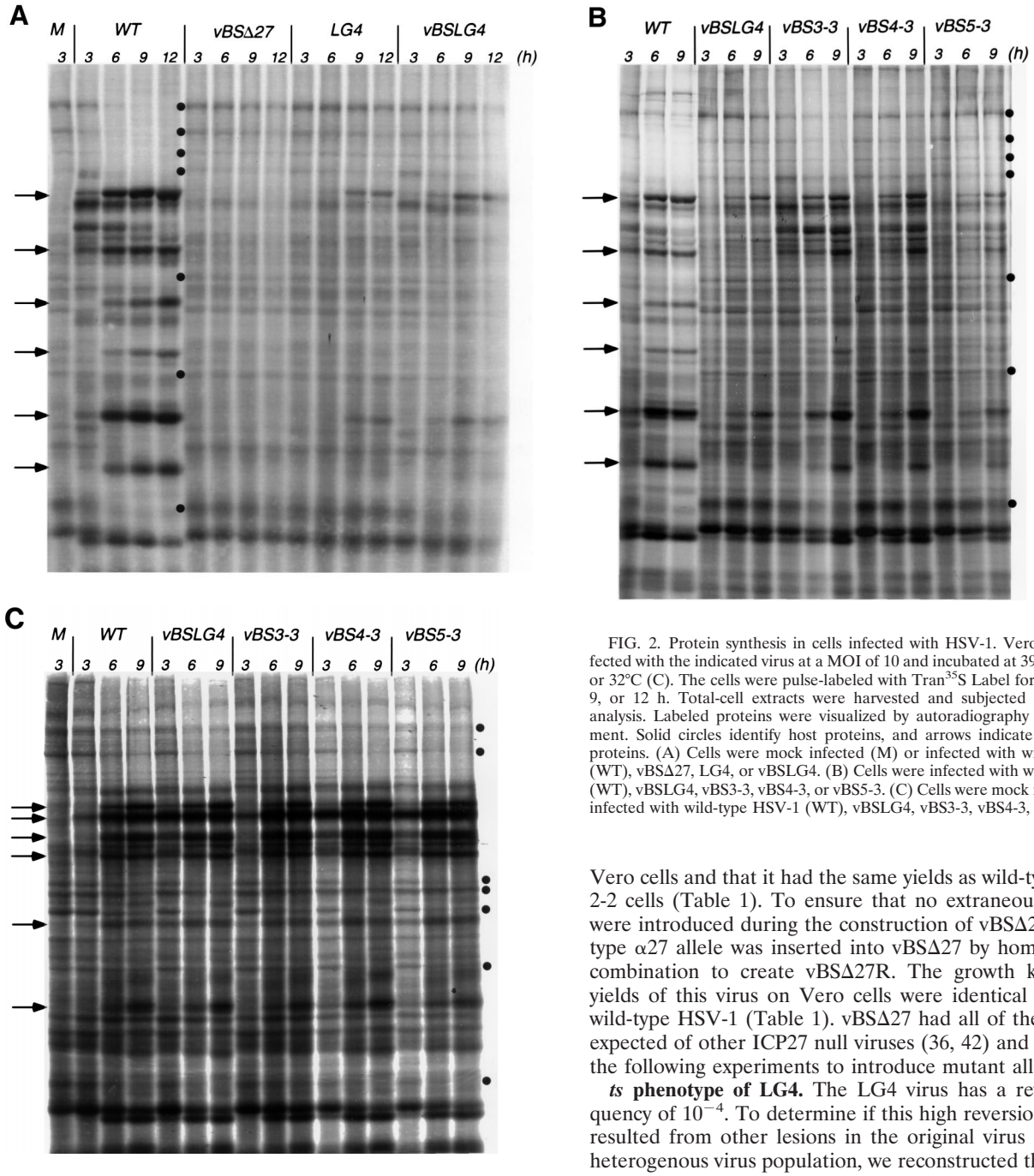

FIG. 2. Protein synthesis in cells infected with HSV-1. Vero cells were infected with the indicated virus at a MOI of 10 and incubated at 39.5°C (A and B) or 32°C (C). The cells were pulse-labeled with Tran^{35}S Label for 30 min at 3, 6, 9, or 12 h. Total-cell extracts were harvested and subjected to SDS-PAGE analysis. Labeled proteins were visualized by autoradiography after enhancement. Solid circles identify host proteins, and arrows indicate virus-specified proteins. (A) Cells were mock infected (M) or infected with wild-type HSV-1 (WT), vBSΔ27, LG4, or vBSLG4. (B) Cells were infected with wild-type HSV-1 (WT), vBSLG4, vBS3-3, vBS4-3, or vBS5-3. (C) Cells were mock infected (M) or infected with wild-type HSV-1 (WT), vBSLG4, vBS3-3, vBS4-3, or vBS5-3.

RESULTS

Construction of an α27 transplacement virus. vBSΔ27, an HSV-1 transplacement virus for α27, was constructed as a template to generate viruses with mutations in ICP27. In this virus, the α27 coding sequence is replaced with a functional *lacZ* gene (Fig. 1). Because ICP27 is essential, the transplacement virus was generated with 2-2 cells (48) to complement the ICP27 deficiency. The replacement of the α27 allele in vBSΔ27 with *lacZ* coding sequences was verified by Southern blot analysis (data not shown). Analysis of the growth and replication of vBSΔ27 revealed that it did not replicate its DNA or grow in Vero cells and that it had the same yields as wild-type virus on 2-2 cells (Table 1). To ensure that no extraneous mutations were introduced during the construction of vBSΔ27, the wild-type α27 allele was inserted into vBSΔ27 by homologous recombination to create vBSΔ27R. The growth kinetics and yields of this virus on Vero cells were identical to those of wild-type HSV-1 (Table 1). vBSΔ27 had all of the properties expected of other ICP27 null viruses (36, 42) and was used in the following experiments to introduce mutant alleles of α27.

***ts* phenotype of LG4.** The LG4 virus has a reversion frequency of 10^{-4}. To determine if this high reversion frequency resulted from other lesions in the original virus isolate or a heterogenous virus population, we reconstructed the LG4 mutation (R480H) in the transplacement virus by homologous recombination, generating vBSLG4. The cloned *ts* α27 allele from LG4 was cotransfected with vBSΔ27 nucleocapsids (49) on 2-2 cells, and recombinants were selected for growth on Vero cells at 32°C. Several independent recombinants were generated, and each failed to form plaques at the restrictive temperature. In contrast, the original stock of LG4 formed plaques at a low frequency at the restrictive temperature (Table 2) (46).

The difference in growth restriction between LG4 and vBSLG4 was examined by testing the plating efficiency of LG4. Independent plaques of LG4 were purified at 32 and 39.5°C. Isolates purified at 32°C were defective for growth at 39.5°C, whereas isolates purified at 39.5°C displayed equivalent plating

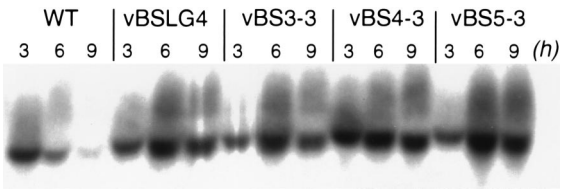

FIG. 3. Northern blot analysis of β-actin mRNA. Vero cells were infected at 39.5°C at a MOI of 10 with wild-type HSV-1 (WT), vBSLG4, vBS3-3, vBS4-3, or vBS5-3. At 3, 6, 9, and 12 h postinfection, the cells were harvested, total RNA was isolated, and 20 μg of each RNA sample was analyzed by gel electrophoresis. The RNA was then transferred to a nylon membrane and probed for β-actin RNA.

efficiencies at both temperatures. Thus, these data demonstrate that the original stock of LG4 contains a small percentage of revertants. We conclude that the LG4 mutation in α27 generates a tight conditionally lethal phenotype.

LG4 revertants contain intragenic suppressors. To determine the nature of the LG4 revertants, individual plaques were isolated from cells infected at 39.5°C. These revertants were plaque purified three times, and their plaque-forming efficiencies at 32 and 39.5°C were determined. Four revertants with plating efficiencies of 1 were further analyzed. The α27 locus from each revertant was cloned, and the nucleotide sequence of the ORF was determined. In all four of the alleles analyzed, the LG4 mutation (R480H) was retained. Each of the revertant alleles also contained an additional point mutation. In two revertants, 2-3 and 4-3, L is substituted for S at amino acid 334. The other revertants, 3-3 and 5-3, contain mutations that result in the conversion of a V to an I at positions 496 and 487, respectively (Fig. 1).

To determine if these additional mutations suppress the *ts* phenotype of LG4, each α27 revertant allele was recombined into the genome of vBSΔ27 (Fig. 1). The plaque-forming efficiency of the resulting viruses (vBS2-3, vBS3-3, vBS4-3, and vBS5-3) at 39.5 versus 32°C remained 1 (Table 2). Therefore, these second-site intragenic mutations in ICP27 suppress the *ts* phenotype of LG4.

Protein synthesis and host cell shutoff in infected cells. Shortly after infection with HSV, host cell protein synthesis is shut off (7, 51). This process reflects the introduction of a virion-associated protein and the expression of ICP27 (10, 12, 27, 28). Because LG4 is defective in host shutoff at 39.5°C, we asked if the revertant viruses shut off cellular protein synthesis at this temperature. Figure 2A shows the pattern of polypeptides synthesized at various times in cells infected with wild-type HSV-1, vBSΔ27, LG4, or vBSLG4. There was a gradual shut off of host cell protein synthesis in cells infected with wild-type HSV-1, and virus proteins were detected by 3 h postinfection. In contrast, the pattern of cell polypeptide synthesis in cells infected with vBSΔ27 was unchanged from 3 to 12 h postinfection, and no virus-specified proteins were detected. The patterns of proteins synthesized in cells infected with LG4 or vBSLG4 were similar. There was no obvious shut off of cellular protein synthesis by these viruses. However, some virus-specified polypeptides were synthesized during infection. These results are consistent with previous results with LG4 (10, 45, 47).

A kinetic analysis of the profile of polypeptides synthesized in cells infected with the revertants vBS3-3, vBS4-3, and vBS5-3 was performed (Fig. 2B). This analysis demonstrates that the revertants synthesize virus-specified proteins but fail to shut off host protein synthesis compared with wild-type HSV-1. While the abundance of individual virus polypeptides

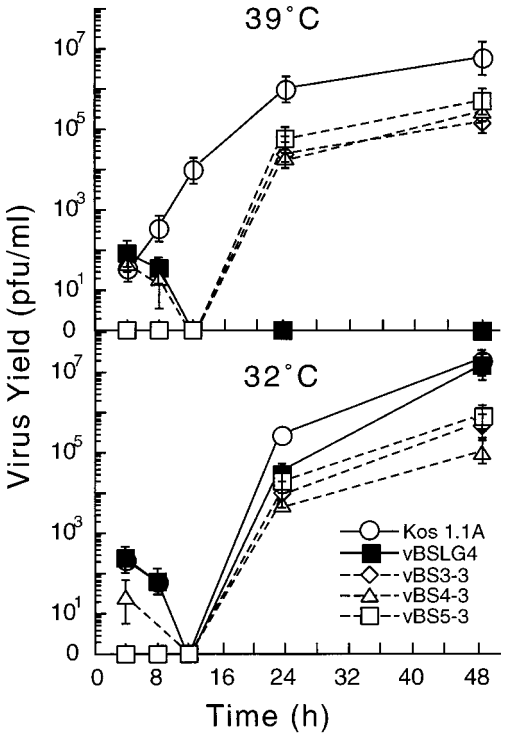

FIG. 4. Analysis of temperature-dependent virus growth. Vero cells were infected at a MOI of 0.1 with wild-type HSV-1, vBSLG4, vBS3-3, vBS4-3, or vBS5-3 at either 39.5 or 32°C, as indicated. The infections were stopped at the indicated times, and the virus yields were determined by titration on Vero cells at 32°C. Data points represent the average of three independent infections, each titrated in duplicate.

is reduced compared to wild-type HSV-1, the revertants synthesized more virus proteins than did vBSLG4. Thus, the intragenic suppressors restore the production of virus proteins yet fail to shut off host protein synthesis.

We also analyzed the proteins synthesized at the permissive temperature and found that vBSLG4 shut off host protein synthesis and synthesized virus-specified polypeptides to wild-type levels (Fig. 2C). However, the revertants showed an intermediate level of shutoff of host protein synthesis, suggesting that this function of ICP27 may be impaired at the permissive temperature. Moreover, the levels of virus protein synthesis in cells infected with the revertants were reduced relative to those in cells infected with wild-type HSV-1 or vBSLG4. The significance of this observation becomes important when interpreting the growth curves of the revertant viruses (see Fig. 4).

ICP27 affects host cell protein synthesis by inhibiting pre-mRNA splicing, which results in a decrease in the accumulation of cellular mRNAs (10, 12). To determine if the revertants also affect host cell pre-mRNA splicing, Northern blot analysis was performed to determine the level of β-actin RNA in cells infected at 39.5°C. Figure 3 demonstrates that there was a rapid decrease of β-actin RNA levels during the course of an infection with wild-type HSV-1. However, β-actin RNA levels were largely unaffected in Vero cells infected with vBSLG4 or the revertants. Therefore, the suppressor mutations do not restore the function of ICP27 that inhibits host cell pre-mRNA splicing during a virus infection at 39.5°C.

Virus growth kinetics. To determine if there were differences in growth rate and/or virus yield, the kinetics of virus production by vBSLG4 and the revertants were compared to

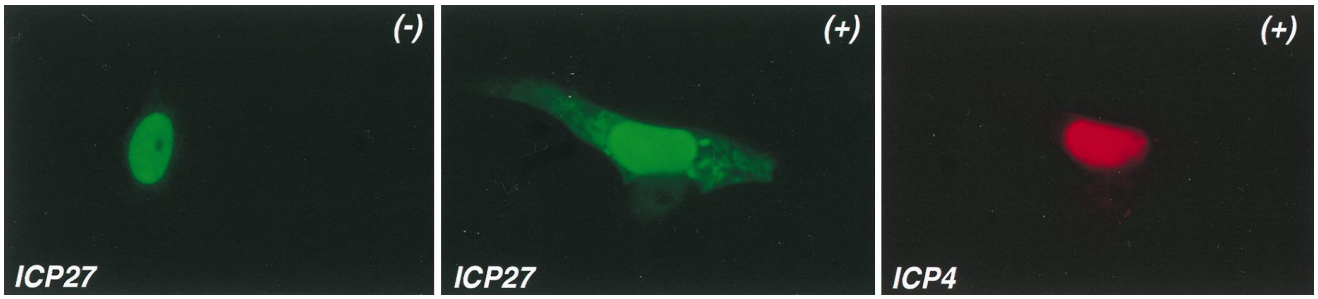

FIG. 5. Intracellular distribution of ICP27 in HSV-1-infected cells. Vero cells were infected at a MOI of 0.01 with wild-type HSV-1 for 12 h and either left untreated (−) or treated with 4 μg of actinomycin D per ml (+) for 1 h. The images show the immunofluorescent staining patterns for ICP27 and ICP4. The images in the two right panels are of the same cell double stained for ICP27 and ICP4 and viewed with filters for the selective visualization of FITC or rhodamine, respectively.

those for wild-type HSV-1. The revertants grew more slowly and their yields were lower than for the wild-type virus at 39.5°C (Fig. 4). vBSLG4 did not grow at 39.5°C. At 32°C, the growth kinetics and yields of vBSLG4 were similar to those of the wild type whereas the yields of the three revertants were decreased. These decreased yields, at both temperatures, presumably result from the decreased synthesis of virus-specified proteins as described above (Fig. 2).

ICP27 shuttles between the nucleus and the cytoplasm. The Rev protein of human immunodeficiency virus (HIV) regulates the transport of unspliced, virus-specified RNAs from the nucleus (6, 21). It contains a nuclear localization signal (NLS) and a nuclear export signal (NES). These signals allow Rev to shuttle between the nucleus and cytoplasm (17, 26, 40). ICP27 localizes to the nucleus, and its major NLS lies between amino acids 109 and 138 (13, 24). ICP27 also contains an amino-terminal leucine-rich region (amino acids 7 to 15) which resembles the NES of Rev (53). These observations led us to ask if ICP27 also shuttles between the nucleus and cytoplasm.

The detection of shuttling proteins is complicated by their failure to accumulate in the cytoplasm. The addition of actinomycin D alters the dynamics of shuttling, resulting in the accumulation of proteins such as Rev and heterogeneous nuclear ribonucleoproteins in the cytoplasm by blocking their reimport into the nucleus (17, 26, 34, 40). Cells infected with HSV-1 were treated with actinomycin D to determine if ICP27 shuttles. In the absence of drug treatment, ICP27 is located predominantly in the nucleus (Fig. 5). At late times postinfection, low levels of ICP27 could be detected in the cytoplasm. When infected Vero cells were treated with actinomycin D at 9 h postinfection, ICP27 could be detected in the cytoplasm of some cells (data not shown). Treatment of infected cells at 12 h resulted in the accumulation of ICP27 in the cytoplasm of most cells (Fig. 5). In contrast, addition of actinomycin D at 6 h postinfection did not result in accumulation of detectable ICP27 in the cytoplasm of Vero cells.

To distinguish between nuclear leakage of ICP27 and a temporal restriction on ICP27 shuttling at late times postinfection, infected Vero cells were stained for both ICP27 and ICP4 (another virus-specified nuclear protein). At 12 h postinfection, ICP27 was detected in the nucleus and the cytoplasm of cells treated with actinomycin D whereas ICP4 was detected only in the nucleus (Fig. 5).

To exclude the possibility that the cytoplasmic accumulation of ICP27 resulted from de novo protein synthesis, the studies described above were repeated in the presence of the protein synthesis inhibitor cycloheximide. Inhibition of protein synthesis did not alter the distribution of ICP27 at late times postinfection (data not shown). Therefore, ICP27 accumulates in the cytoplasm as a consequence of shuttling and not de novo protein synthesis. Accordingly, the remaining experiments were performed without cycloheximide.

This series of experiments demonstrates that shuttling of ICP27 is restricted to late times postinfection and that its appearance in the cytoplasm results from nuclear export and not breakdown of the nuclear envelope. This temporal change in the intracellular localization of ICP27 coincides with the requirement for ICP27 for the synthesis of late proteins. Thus, shuttling may contribute to the expression of late proteins.

A *trans*-acting cofactor is required for ICP27 to shuttle. The previous experiments were done in the context of a virus infection. To determine if ICP27 shuttled in the absence of other HSV-1-specified proteins, a transient-expression assay was developed. Vero cells were transfected with an ICP27 expression construct, and the intracellular localization of ICP27 was examined in the presence or absence of actinomycin D. Under either of these conditions, ICP27 was found to accumulate only in the nucleus of transfected Vero cells (Fig. 6). The results of this experiment and the demonstration that ICP27 shuttles in infected cells imply that ICP27 requires a cofactor to shuttle.

The presence of an RGG box (RNA-binding domain) in ICP27 (25) and the ability of ICP27 to shuttle at late times postinfection suggested that nuclear export of this protein might be dependent on the presence of virus-specified late RNAs. To determine if the presence of a late virus-specified RNA affected ICP27 shuttling, Vero cells were cotransfected with plasmids that express α27 and a late-gene (UL48) RNA. Under this condition, ICP27 was detected in the cytoplasm of cotransfected cells treated with actinomycin D (Fig. 6). To determine if the UL48 RNA or its translation product (α-Tif) served as the cofactor for ICP27 shuttling, two additional plasmids were constructed. In plasmid pBS3term, an oligonucleotide with stop codons in all three reading frames was inserted into the UL48 ORF at nucleotide +582 relative to the ATG. In the second plasmid (pBSΔSac), the sequences encoding amino acids 1 to 423 of α-Tif were deleted, leaving 204 bp of coding sequence and the 3′ untranslated region of UL48. In cells cotransfected with either plasmid, ICP27 accumulated in the cytoplasm following treatment with actinomycin D (Fig. 6). The use of these two constructs eliminates a role for the UL48 protein in shuttling. Therefore, transcription of the terminal 204 nucleotides and the 3′ untranslated region of the UL48 RNA is sufficient to facilitate shuttling of ICP27 in transfected Vero cells.

To determine the specificity of the cofactor requirement of ICP27, we tested whether ICP27 would shuttle in the presence

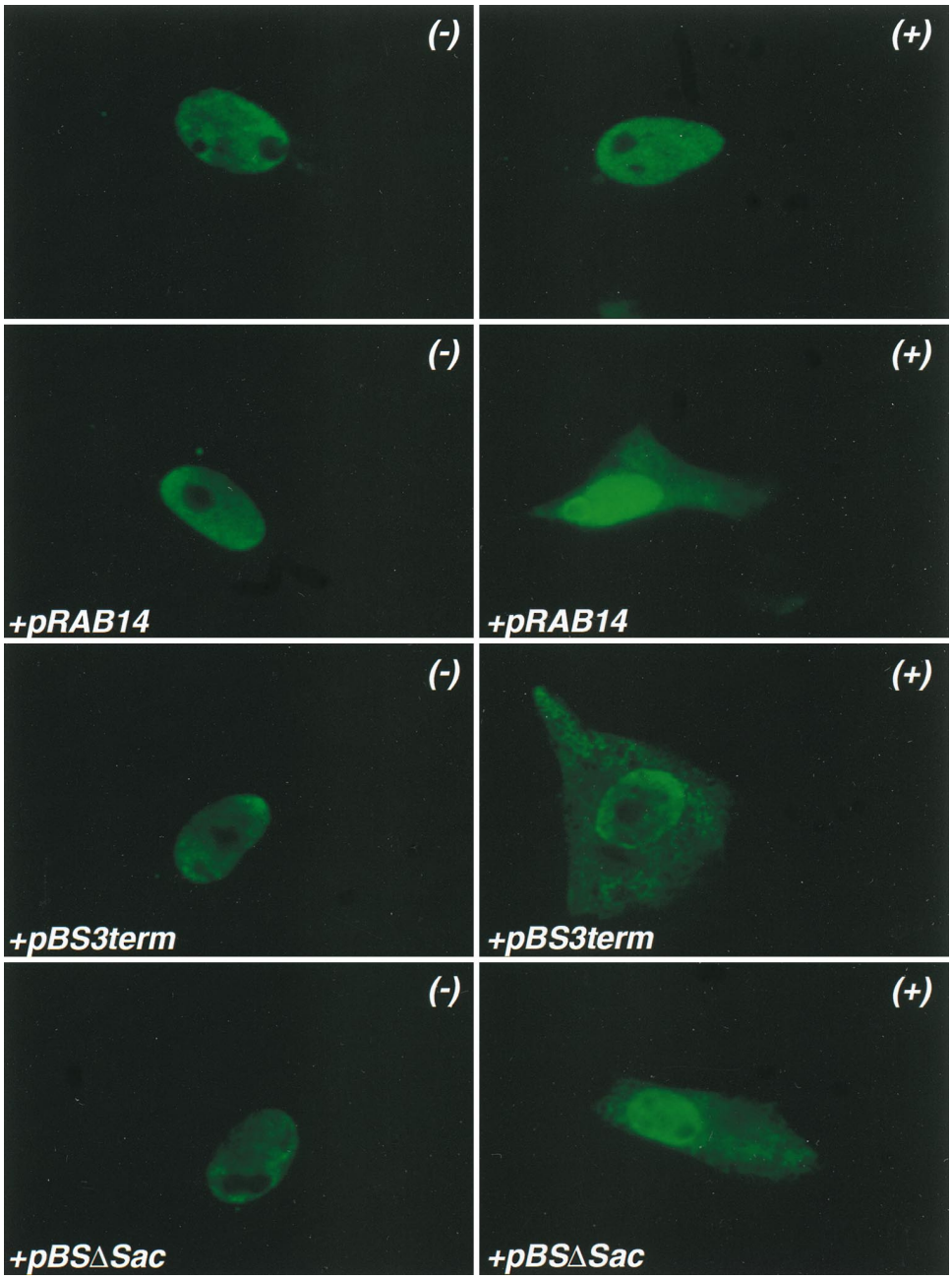

FIG. 6. Intracellular distribution of ICP27 in transfected cells. Vero cells were transfected with pBS27 expressing ICP27 or cotransfected with pBS27 and either pRAB14 (UL48), pBS3term, or pBSΔSac as indicated. At 48 h posttransfection, the cells were treated with 4 μg of actinomycin D per ml (+) for 1 h or left untreated (−). The intracellular distribution of ICP27 was examined with an antibody specific for ICP27, as described in Materials and Methods.

of RNA from an α gene. We note that α27 RNA is present in cells transfected with only an ICP27 expression plasmid; however, neither this RNA nor the resident cell RNAs serve as cofactors for ICP27 to shuttle in transfected Vero cells. Moreover, when cells were cotransfected with plasmids expressing α0 and α27 RNAs, ICP27 was found only in the nucleus after treatment with actinomycin D (data not shown). These data suggest that shuttling of ICP27 is dependent on a cofactor and that at least one late-gene RNA can act as this cofactor.

ICP27 shuttling correlates with the appearance of late proteins. In cells infected with LG4 at the nonpermissive temperature, γ proteins do not accumulate (47) (Fig. 2). This defect in accumulation of late proteins appears to occur at the posttranscriptional level since the rates of γ gene transcription in cells infected with LG4 or wild-type virus are equivalent (47). Therefore, to determine the relationship between shuttling and synthesis of γ proteins, the nuclear export of ICP27 during an infection with vBSLG4 was studied. Figure 7 shows that at late times postinfection, *ts*ICP27 shuttled at the permissive temperature but not at the restrictive temperature. This demonstrates that ICP27 from vBSLG4 is *ts* for shuttling and hence that shuttling correlates with the appearance of late proteins.

To determine if the defect in *ts*ICP27 resulted from the

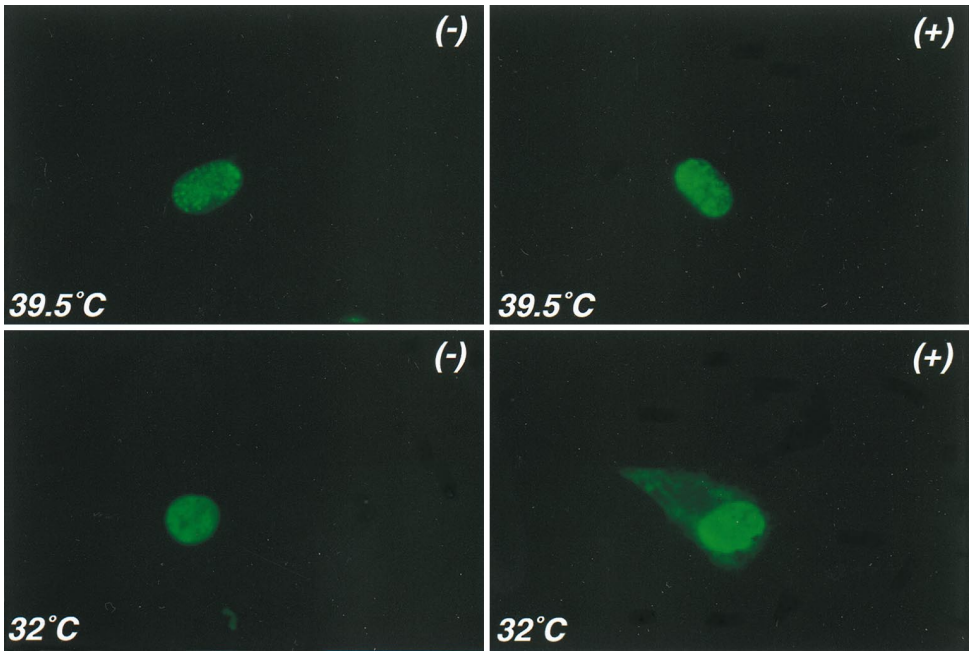

FIG. 7. Intracellular distribution of ICP27 in cells infected with vBSLG4. Vero cells were infected at a MOI of 0.01 with vBSLG4 for 12 h at 39.5 or 32°C, as indicated. Infected cells were either left untreated (−) or treated with 4 μg of actinomycin D per ml (+) for 1 h. The intracellular distribution of ICP27 was examined with an antibody specific for ICP27.

failure to shuttle at the nonpermissive temperature or if it reflected an earlier event, a temperature shift experiment was performed. Vero cells were infected with vBSLG4 at either 39.5 or 32°C for 12 h and then shifted to the permissive or nonpermissive temperatures for 1 h. The infected cells were then either treated with actinomycin D for 1 h or left untreated, and the subcellular distribution of ICP27 was examined. Figure 8 demonstrates that following a shift down, *ts*ICP27 accumulated in the cytoplasm of cells treated with actinomycin D. The control shift up experiment reveals that the ability of *ts*ICP27 to shuttle was severely limited. Thus, *ts*ICP27 synthesized at 39.5°C can attain the proper conformation and shuttle when the infected-cell cultures are shifted to the permissive temperature.

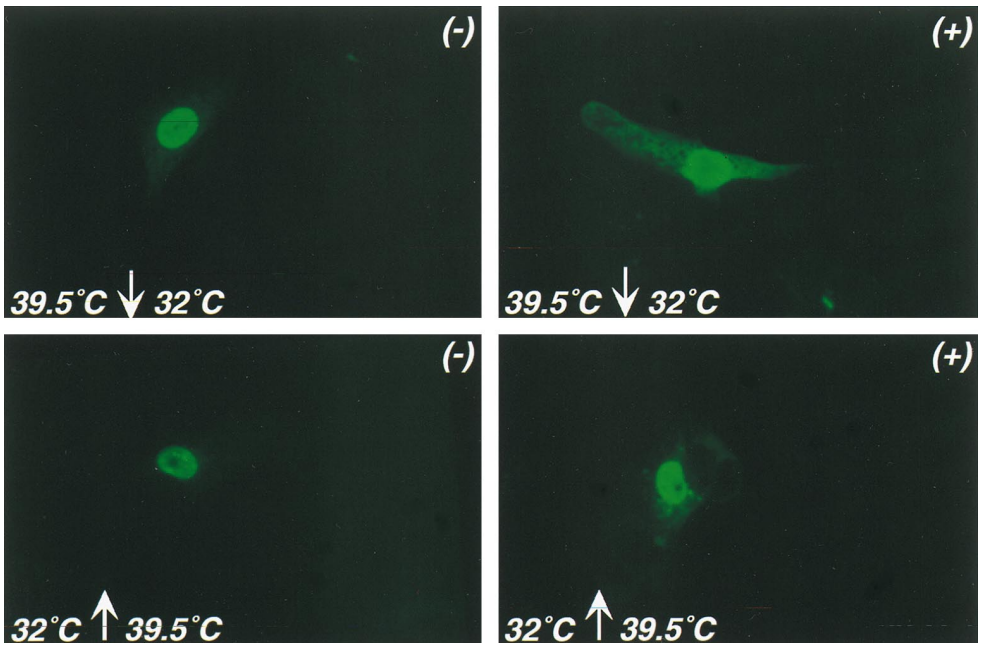

FIG. 8. Intracellular distribution of ICP27 after a temperature shift in cells infected with vBSLG4. Vero cells were infected with vBSLG4 at a MOI of 0.01 at either 39.5 or 32°C. At 12 h postinfection, the cells were shifted to 32 or 39.5°C, respectively, for 1 h, at which time they were either left untreated (−) or treated with 4 μg of actinomycin D per ml (+) for 1 h. The intracellular distribution of ICP27 was examined with an antibody specific for ICP27.

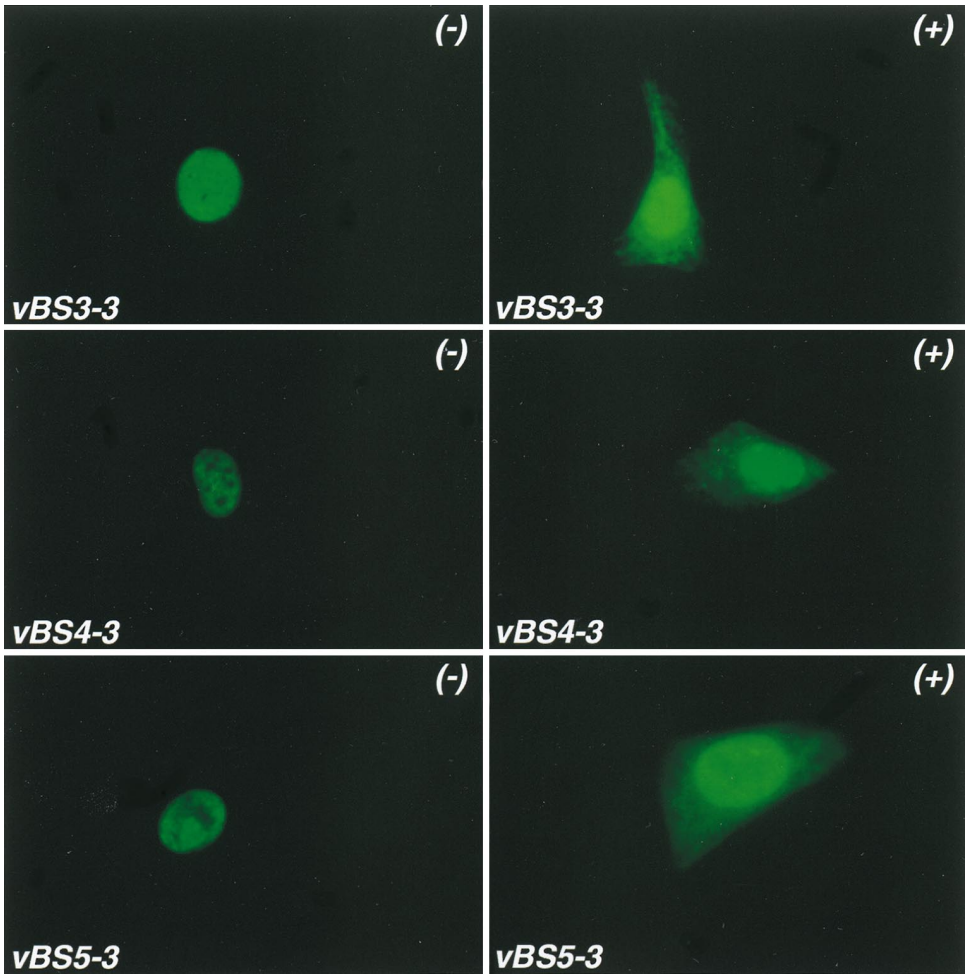

FIG. 9. Intracellular distribution of ICP27 in cells infected with revertants of LG4. Vero cells were infected at a MOI of 0.01 with either vBS3-3, vBS4-3, or vBS5-3, as indicated, for 12 h at 39.5°C. Infected cells were either left untreated (−) or treated with 4 μg of actinomycin D per ml (+) for 1 h. The intracellular distribution of ICP27 was examined with an antibody specific for ICP27.

We next asked if the intragenic suppressors of LG4 rescue shuttling. In Vero cells infected with the revertant viruses, ICP27 shuttled at both 39.5°C (Fig. 9) and 32°C (data not shown). Thus, the suppressors restore ICP27 shuttling in HSV-1-infected cells. These data and the finding that the suppressors restore growth support the conclusion that shuttling is important for virus growth. Therefore, we propose that one function of ICP27 is to regulate the production of late proteins by mediating the nucleocytoplasmic export of HSV-1 late RNAs.

DISCUSSION

Intragenic suppressors of a *ts* allele of α27, an essential HSV-1 regulatory protein, separate two functions of ICP27 that were disrupted by the original mutation. This *ts* phenotype results from a single amino acid substitution at position 480 and renders HSV-1 defective for both the shutoff of host protein synthesis and the synthesis of late virus proteins (10, 47). The failure to synthesize γ proteins prevents virus growth. Revertants of LG4 were selected for growth at 39.5°C, and three unique second-site intragenic suppressors were identified. While these revertants restore the synthesis of late proteins and virus growth, they fail to inhibit the splicing of cellular RNAs. This demonstrates that host shutoff is not required for virus growth and separates this function of ICP27 from its role in late-protein synthesis.

The inability of the revertants to shut off host protein synthesis correlates with decreases in both virus protein synthesis and yield (Fig. 2 and 3). These observations suggest that the inhibition of host cell pre-mRNA splicing by ICP27 results in selective synthesis of virus-specified proteins and thus increased virus production, since most virus-specified transcripts are not spliced.

The role of ICP27 in HSV-1 late gene production was also investigated. We observed that ICP27 shuttles between the nucleus and the cytoplasm only at late times postinfection. This event correlates with the role of ICP27 in the production of late proteins. Other RNA binding proteins that shuttle do so continuously and are thought to be involved in RNA transport (17, 26, 40). HIV Rev, an RNA binding protein that shuttles, mediates the export of unspliced HIV RNAs and is required for the expression of a subset of HIV genes (6, 21). We propose that ICP27 plays a similar role in regulating the expression of late-gene transcripts.

Shuttling of ICP27 is temporally regulated in infected cells. However, ICP27 does not shuttle when transiently expressed in Vero cells. This difference led us to postulate that ICP27 re-

quires a cofactor to shuttle. This requirement was satisfied by coexpression with a plasmid expressing the late gene UL48. Because ICP27 has been shown to bind RNA through its RGG box (23), we presume that the interaction between ICP27 and UL48 RNA facilitates its export from the nucleus. This observation and the ability of ICP27 to discriminate between late and immediate-early RNAs in a transient-expression assay suggest that ICP27 can differentiate in vivo between cellular RNAs and classes of virus RNAs.

The requirement for ICP27 in the expression of late-gene proteins suggests that ICP27-mediated export of late RNAs may be important for their subsequent expression. This idea is supported by the temporal correlation between shuttling and the synthesis of late proteins. Furthermore, nuclear run-on experiments reveal no difference in the rate of transcription from late genes in cells infected with LG4 or wild-type virus at 39.5°C (47). These results suggest that the defect in LG4 late-protein synthesis is a consequence of the failure of *ts*ICP27 to process virus-specified RNAs at the posttranscriptional level. Here, we show that *ts*ICP27 in cells infected with LG4 at 39.5°C does not shuttle. However, protein synthesized at this temperature has the capacity to shuttle when infected cells are shifted down. Moreover, the shift-down experiment suggests that the defect in shuttling seen with *ts*ICP27 does not result from an upstream event but, rather, reflects a structural requirement. The shift-up experiment suggests that synthesis of late RNAs per se is not sufficient to trigger shuttling. In contrast, the ICP27s expressed by the revertants of LG4 shuttle and rescue late-protein synthesis. Thus, shuttling can be linked to late-gene expression.

The correlation between the ability of ICP27 to shuttle and its requirement for late-protein synthesis leads us to propose a model to explain why ICP27 shuttling is restricted to late times postinfection. We propose that binding of late RNAs by the RGG box of ICP27 either exposes the NES or masks the adjacent NLS, permitting export of this protein from the nucleus. Once in the cytoplasm, the associated RNA is released from ICP27, thereby unmasking the NLS. These events would permit the reimport of ICP27 into the nucleus, thus completing the shuttling cycle. These processes would couple the shuttling of ICP27 with the export of late RNAs and result in their translation. Parenthetically, support for this model comes from the demonstration that the RGG box is required to activate γ gene expression (13, 24). The requirement for a virus-encoded RNA export protein may stem from either a shutoff of host-encoded export pathways or an inherent requirement for an alternative pathway, since the majority of virus RNAs are not spliced. Cellular transcripts which undergo splicing may utilize an RNA export pathway that is not accessed by intronless RNAs.

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