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The Herpes Simplex Virus Regulatory Protein ICP27 Contributes to the Decrease in Cellular mRNA Levels during Infection

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We have previously shown that the herpes simplex virus immediate-early regulatory protein ICP27 acts posttranscriptionally to affect mRNA processing (R. M. Sandri-Goldin and G. E. Mendoza, Genes Dev. 6:848–863, 1992). Specifically, in the presence of ICP27, spliced target mRNAs were decreased 5- to 10-fold in transfections with target genes containing a 5′ or 3′ intron. Here, we have investigated the effect of ICP27 during herpes simplex virus type 1 (HSV-1) infection on accumulation of spliced cellular mRNAs. ICP27 viral mutants have been shown to be defective in host shutoff (W. R. Sacks, C. C. Greene, D. P. Aschman, and P. A. Schaffer, J. Virol. 55:796–805, 1985). Therefore, we examined whether ICP27 could contribute to this complex process by decreasing cellular mRNA levels through its effects on host cell splicing. It was found that in infections with viral mutants defective in ICP27, the accumulated levels of three spliced host mRNAs were higher than those seen with wild-type HSV-1. The differences occurred posttranscriptionally as shown by nuclear runoff transcription assays. The stabilities of the spliced products during infection with wild-type or ICP27 mutant viruses were similar, and unspliced precursor mRNA for a viral spliced gene was detected in infections with wild-type HSV-1 but not in infections in which ICP27 was not expressed. These results suggest that the reduction in cellular mRNA levels and the accumulation of pre-mRNA are related and may be caused by an impairment in host cell splicing. These data further show that ICP27 is required for these effects to occur.

Lytic infection with herpes simplex virus results in the repression of most host cell protein synthesis (7, 8, 10, 18, 22, 23, 32–34, 37, 48, 55, 57, 58) and the sequential expression of at least four classes of coordinately regulated viral genes (43). The inhibition of host protein synthesis is a multiphase process. The shutoff is initially caused by a structural component of the infecting virion which causes disaggregation of cellular polyribosomes (57, 58) and degradation of host mRNAs (37, 48, 55). Isolation of mutants defective in this virion host shutoff (*vhs*) activity has enabled this function to be mapped to coordinates 0.604 to 0.606 on the viral genome (23). The open reading frame in this region, designated UL41, encodes a protein with an apparent molecular mass of 55 kDa (9, 23, 28). The *vhs* protein acts by facilitating the nonspecific degradation of host mRNAs and also viral mRNAs (21, 34, 37). A delayed or secondary stage of host shutoff reduces the remaining levels of host protein synthesis and requires prior viral gene expression (11, 22, 33, 37, 55). The protein(s) responsible for the secondary shutoff has not been identified.

It has previously been reported that temperature-sensitive (*ts*) and deletion mutants with mutations in the immediate-early regulatory protein ICP27 (IE63 and UL54) were defective in their ability to shut off host protein synthesis (44). Viral mutants defective in ICP27 display a variety of phenotypes, including an overexpression of some immediate-early and early gene products, a decrease in DNA synthesis, an underexpression of late gene products, and an impairment in host shutoff (27, 40, 41, 44, 52). We have been studying the mode of action of ICP27. Transfection experiments have demonstrated that ICP27 can activate or repress expression, depending on the target gene (1, 17, 30, 39, 42, 50, 56). However, the regulatory activity of ICP27 is independent of the target gene promoter sequences; instead, it depends on the presence of different mRNA processing signals (5, 29, 47). Particularly striking was the observation that spliced target mRNAs were decreased 5- to 10-fold in transfections with target genes containing a 5′ or 3′ intron (47). This result suggests that ICP27 may affect host cell splicing. Because the majority of herpes simplex virus type 1 (HSV-1) transcripts are not spliced (60), whereas the majority of cellular transcripts are spliced, we have considered the possibility that ICP27 may contribute to the shutoff of host protein synthesis by interfering with efficient splicing of host cell mRNAs, which would result in a decrease of spliced products presented to the translation machinery.

We have begun to test this hypothesis by analyzing the levels of spliced cellular mRNAs during HSV-1 infection. Here, we show that in infections with viral mutants defective in ICP27, the accumulated levels of at least three spliced host mRNAs were higher than those seen with wild-type infected cells. Furthermore, the differences seen between infections with HSV-1 wild-type and ICP27 mutant viruses occurred posttranscriptionally and did not result from altered stabilities of the spliced products. ICP27 appeared to be the only viral gene product required to cause this decrease in the amounts of spliced mRNAs. Finally, unspliced precursor mRNA was found in HSV-1 KOS1.1 infections, further suggesting that host cell splicing may be impaired when ICP27 is functional.

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MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells, HeLa R19 cells, and rabbit skin fibroblast cells were grown as described previously (50, 53). The cell line 2-2, which contains the wild-type ICP27 gene; *Stu15*, which contains the ICP27 mutant S1B (17); and *Sst26*, which contains the ICP27 mutant S23 (17), have been described previously (52, 54). HSV-1 KOS 1.1, the ICP27 *ts* mutant *ts*LG4 (44, 46), and the mutant 27-LacZ, which has an insertion of the *lacZ* gene in the ICP27 locus (52), were propagated as described previously (46, 52). Virus 27R was isolated by marker rescue of the chimeric ICP27-*lacZ* gene in the mutant 27-LacZ by cotransfection of 27-LacZ DNA with *Eco*RI fragment EK isolated from pSG28 (14), which contains the wild-type ICP27 gene. Progeny virus which had a white plaque phenotype after treatment with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), failed to hybridize with a probe specific for the *lacZ* gene, and were able to grow on Vero cells were further analyzed by Southern blot hybridization analysis to confirm the presence of the wild-type ICP27 gene. One such isolate was termed 27R and used in this study. For UV inactivation of KOS 1.1 and 27-LacZ (see Fig. 5 and 6), virus stocks were suspended in phosphate-buffered saline (PBS) at a concentration of 2×10^7 PFU/ml. A 5-ml amount of the virus suspension in a 100-mm-diameter culture dish was irradiated with a germicidal lamp (wavelength, 254 nm) for 10 min with constant agitation. The incident radiation at the surface of the suspension was 15 ergs/s/mm^2. Virus titers were reduced greater than 5 logs by this treatment.

Transfections. Rabbit skin fibroblast cells were grown as described previously (50). Transfections were performed in 100-mm-diameter dishes as described previously (50). RNA was isolated 36 h after transfection. In the experiment shown in Fig. 9, actinomycin D was added to some of the transfected cultures as indicated at a concentration of 10 μg/ml 36 h after transfection. RNA was isolated 6 h later. Plasmids pSG28K/B, which encodes ICP4; pRS-1, which encodes ICP0; pSG130B/S, which encodes ICP27; and pB7, which contains an ICP27 gene with an insertion in the repressor region have been described previously (17, 50). Plasmids pSV2-CAT (16) and pTK-CAT-5′S-SVLPA (47) were added to transfections as target plasmids as described previously (47). Plasmids pCMV-27 and pCMV-B7, which contain the promoter and enhancer of an immediate-early gene of human cytomegalovirus (2), were constructed by inserting a *Pst*I-*Sst*I fragment from plasmid pCMVβ (Clonetech) into a *Hin*fI site 55 bp upstream of the translational start of ICP27 after removal of the ICP27 promoter sequences and appropriate modification of the ends. The plasmid pCMV-β-Gal was derived from pCMVβ by deletion of an *Xho*I-*Not*I fragment to remove the simian virus 40 (SV40) splicing signals. The plasmid pUL41 was subcloned into pUC18 as a 3.8-kb *Hin*dIII-*Sal*I fragment from pSG124 (14) as described by Read et al. (38).

Northern (RNA) hybridizations. Total RNA was extracted at the times indicated for each experiment by the guanidium thiocyanate method (4). Cytoplasmic RNA was isolated as described previously (47). RNA was quantified by optical density readings. Equivalent amounts of RNA (usually 20 μg) were denatured in glyoxal (59) and fractionated on 1% agarose gels. To confirm that equivalent amounts of RNA were analyzed, portions of each sample loaded on glyoxal gels were fractionated in agarose and stained with ethidium bromide to visualize the 28S and 18S rRNAs. Gels were photographed with positive/negative film, and the negatives were scanned by laser densitometry. Photographs of the negatives are shown below (see Fig. 5 and 7A). As an alternative means to ensure even loading of RNA samples, Northern blots were hybridized with a probe specific for 28S rRNA after hybridization with probes specific for cellular or viral mRNAs. Hybridization with 28S rRNA is shown below (see Fig. 8). Transfer of RNA to GeneScreen (DuPont, NEN Research Products) and hybridization conditions were as described previously (45). The probe used to detect β-tubulin RNA was derived from a plasmid containing a 250-bp *Bam*HI-*Eco*RI fragment from the 3′ untranslated region of a human β-tubulin cDNA clone (24) and was obtained from the American Type Culture Collection. This fragment was cloned into the in vitro transcription vector pGEM-1 (Promega). The β-tubulin probe hybridized with two β-tubulin mRNA species of 1.8 and 2.6 kb which result from alternative polyadenylation sites (24). The probe used to detect β-actin mRNA consisted of a 250-bp portion of a cDNA clone of mouse β-actin which was inserted behind a T7 promoter in an in vitro transcription vector (Ambion). The probe used to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was a 1.3-kb cDNA fragment derived from the plasmid pGAPDH and was obtained from Eric Stanbridge (University of California, Irvine). The 28S rRNA probe was subcloned into pGEM-2 (Promega) as a 1,437-bp *Bam*HI fragment from plasmid pHrA (15) and was transcribed with T7 polymerase. Probes used in Northern hybridization analysis of viral mRNA specific for ICP4, ICP27, thymidine kinase (TK), and glycoprotein C (gC) were as described previously (54). The probe specific for the US11 gene, which also hybridizes with the ICP47 gene, was as described by Johnson and Everett (19). ^{32}P-labeled DNA probes were prepared by random priming (Pharmacia). ^{32}P-labeled RNA probes were prepared by in vitro transcription with T7 RNA polymerase.

Nuclear runoff assays. Vero cells were either mock infected or infected with KOS 1.1 or *ts*LG4 at a multiplicity of infection of 10 at 39.5°C. At 5 or 7 h after infection as indicated (see Table 1), nuclei were isolated from 2.5×10^7 cells for each sample as described by Konieczny and Emerson (20). Nuclear transcription reactions were carried out as described by Weinheimer and McKnight (61) with the conditions described previously (52). The plasmid pβ-tub containing the 250-bp β-tubulin DNA fragment in pGEM-1 was used in in vitro transcription reactions to generate the complementary (antisense) and noncomplementary (sense) strands of RNA by transcribing with T7 and SP6 polymerases. The plasmid containing the 250-bp β-actin probe was similarly used to generate β-actin-specific RNA. Purified transcribed RNA, either complementary or noncomplementary, was denatured in glyoxal and applied to Gene-Screen filters in 0.1- and 1.0-μg amounts with a slot blot apparatus (Bethesda Research Laboratories). Filters were baked at 80°C under vacuum for 2 h and then were prehybridized as described previously (52). RNA samples from the nuclear runoff reactions were heated at 95°C for 5 min and then added to the prehybridization solution. Hybridizations were carried out as described previously (52) at 45°C for 18 h. Filters were washed and treated with RNase A as described previously (52). Filters were exposed to X-ray film and then quantified by liquid scintillation counting of the slots. Background values obtained with the noncomplementary strand were similar to those found with filters to which RNA from vector sequences alone was bound. The values for each probe were subtracted before relative transcription rates were calculated.

RNase protection analysis. Total RNA samples, purified as described previously (4), were treated with RNase-free DNase I (Promega) in the presence of RNasin for 60 min at 37°C to ensure that any residual viral DNA had been removed from the

samples before the RNase protection assays were performed. To generate an ICP0 intron-exon-spanning antisense RNA probe, a T7 expression plasmid in which a 320-bp *Bam*HI-*Ava*I fragment was inserted into pGEM-2 after appropriate modification of the ends was cloned. The plasmid was linearized with *Ear*I before transcription with T7 polymerase. This allowed transcription of a 375-nucleotide antisense RNA including about 50 nucleotides of vector sequence so that the undigested probe could be distinguished from fully protected pre-mRNA. A second ICP0 probe, which consisted of a 435-bp *Bam*HI-*Xho*I fragment from the ICP0 gene inserted into pGEM-2 (Promega), was also used. This allowed transcription of a 476-nucleotide RNA probe. The fully protected ICP0 product (precursor mRNA) was expected to be 435 nucleotides, from the *Bam*HI site in intron 1 to the *Xho*I site in exon 2 (35). Inclusion of 41 nucleotides of additional transcribed sequence from the transcription vector enabled the distinction between undigested probe (476 nucleotides) and fully protected product (435 nucleotides) to be made. The chloramphenicol acetyltransferase CAT RNase protection probe has been described previously (47). A probe antisense to a portion of the ICP27 gene was obtained by cloning a *Sal*II-*Bam*HI fragment into pGEM-2. The plasmid was linearized with *Nco*I, and T7 transcription was performed to generate a 217-nucleotide antisense RNA probe. The ICP4 probe used in the protection assays was cloned as a 1,690-bp *Bam*HI-*Sal*I fragment in pGEM-1. The plasmid was linearized with *Stu*I and transcribed with T7, which resulted in a 349-nucleotide antisense RNA probe specific for ICP4 mRNA. Equal amounts of RNA samples (usually 50 μg) were mixed with 5×10^5 cpm of antisense RNA which had been gel purified. Hybridizations and RNase protections were performed as described by Zinn et al. (63), except that the hybridization temperature was raised to 58°C for hybridization with HSV-1 RNA because of the high GC content. Protected fragments were fractionated on 5% denaturing polyacrylamide-urea gels.

Immunofluorescent staining. Vero cells or 2-2 cells were infected with KOS1.1, 27-LacZ, or UV-inactivated KOS1.1 as indicated in the legend to Fig. 6. Five hours after infection, cells were fixed in 3.7% formaldehyde. Cells were permeabilized in PBS containing 0.5% Nonidet P-40 for 5 min. Cells were rinsed three times in PBS containing 1% newborn calf serum. In general, coverslips were rinsed three times in PBS containing 1% newborn calf serum before the addition of each antibody or reagent. Cells were treated with a 1:100 dilution of donkey serum for 30 min to block nonspecific interactions. Cells were then reacted with the primary antibody for 30 min. Monoclonal antibodies 1112 to ICP0, 1113 to ICP27, 1114 to ICP4, and 1115 to ICP8 were obtained from the Goodwin Institute for Cancer Research. These antibodies were used at dilutions of 1:500. Biotinylated goat anti-mouse antibody (Amersham) at a 1:100 dilution was added to cells for 30 min. Following rinsing, a 1:100 dilution of streptavidin-conjugated fluorescein (Amersham) was added to cells for 30 min. Cells were rinsed three times in PBS containing newborn calf serum and then three times in PBS without serum. Coverslips were mounted in PBS. Cells were examined with a Nikon UFX-II epifluorescence microscope with a 100×, 1.25-numerical-aperture objective lens.

RESULTS

Levels of three cellular mRNAs are reduced in infections with HSV-1 KOS 1.1 compared with those of ICP27 *ts* mutant-infected cells. Studies by Sacks et al. (44) showed that cells infected with four different ICP27 *ts* mutants exhibited greatly impaired shutoff of host protein synthesis. To investigate the basis for this effect, we began by measuring the steady-state levels of three cellular mRNAs in wild-type and ICP27 mutant infections. Specifically, the amounts of β-tubulin, β-actin, and GAPDH mRNAs were determined by Northern blot hybridization of total RNA isolated at various times from HeLa cells which were either mock infected or infected with HSV-1. Experiments were performed at 39.5°C with HSV-1 wild-type strain KOS1.1 and the ICP27 *ts* mutant *ts*LG4 (44, 46). Twenty micrograms of total RNA from each sample was analyzed. As discussed in Materials and Methods, the β-tubulin probe hybridized with two mRNA species of 1.8 and 2.6 kb which result from utilization of alternative polyadenylation sites (24). The results demonstrate that as the infection progressed, the level of each mRNA in KOS 1.1-infected cells decreased significantly compared with the level in *ts*LG4-infected cells (Fig. 1). The amount of each cellular mRNA in *ts*LG4-infected cells also was reduced compared with that of mock-infected controls, and this decrease was likely due to the action of the *vhs* product. However, accumulation of all three mature mRNAs, β-tubulin, β-actin, and GAPDH, was reduced to a greater extent in KOS 1.1-infected cells when ICP27 was functional.

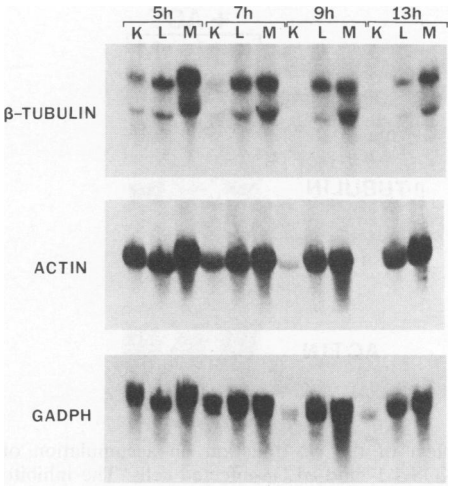

FIG. 1. Northern blot hybridization analysis of cellular mRNAs after infection with HSV-1 KOS 1.1 or *ts*LG4. HeLa cells were mock infected (lanes M) or infected with HSV-1 KOS 1.1 (lanes K) or *ts*LG4 (lanes L) at a multiplicity of infection of 10 at 39.5°C. Total RNA was isolated at 5, 7, 9, and 13 h after infection. Equal amounts of RNA for each sample (20 μg) were fractionated in agarose and transferred to GeneScreen filters. The blots were hybridized with ^{32}P-labeled DNA probes, generated by random priming, which were specific for β-tubulin, β-actin, or GAPDH (see Materials and Methods).

The activity of the input *vhs* function in wild-type- and ICP27 mutant-infected cells is equivalent. One possibility which could explain the differences in cellular mRNA levels between KOS 1.1-infected cells and *ts*LG4-infected cells would be differences in the activity of the virion factor *vhs* (UL41). To determine if the *vhs* functions in KOS 1.1 and *ts*LG4 virions were equivalent, HeLa cells were treated with actinomycin D to inhibit transcription and then infected with KOS 1.1 or *ts*LG4 in the presence of the inhibitor. Under these conditions, transcription of the viral genome is blocked and only proteins present in the virion can function in infected cells. Total RNA was isolated 5 h after infection and analyzed by Northern blot hybridization. As can be seen in Fig. 2, the amounts of

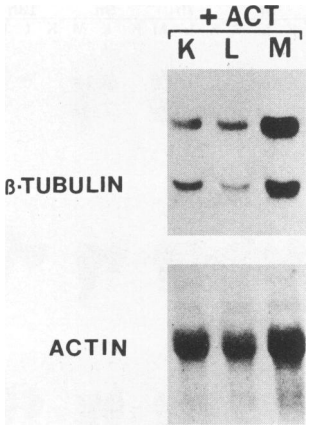

FIG. 2. Effect of the *vhs* function on accumulation of cellular mRNAs in KOS 1.1- and *ts*LG4-infected cells. The inhibitor actinomycin D (10 μg/ml) (+ ACT) was added to HeLa cells which were then mock infected (lane M) or infected with KOS 1.1 (lane K) or *ts*LG4 (lane L) at a multiplicity of infection of 10. Five hours after infection, total RNA was isolated and analyzed by Northern blot hybridization with ^{32}P-labeled DNA probes specific for β-tubulin and β-actin mRNA.

β-tubulin and β-actin mRNAs found in KOS 1.1- and *ts*LG4-infected cells were the same and were significantly reduced from that seen in uninfected controls. In both KOS 1.1- and *ts*LG4-infected cells, smaller amounts of β-tubulin and β-actin mRNAs were seen than were found at the 5-h time point in Fig. 1. The lower levels of cellular mRNAs in Fig. 2 than in Fig. 1 are due to the blocking of transcription by actinomycin D, which was added before infection. Data shown in Fig. 2 indicate that the *vhs* products in KOS 1.1 and *ts*LG4 virions function equivalently during infection and, further, that another viral product not expressed in the presence of actinomycin D was responsible for the differences in the accumulation of host mRNAs in KOS 1.1-infected cells (Fig. 1).

Transcription of β-actin and β-tubulin in KOS 1.1- and *ts*LG4-infected cells is similar. In addition to degrading host cell mRNAs through the action of the *vhs* protein, HSV-1 infection has been shown to inhibit the transcription of some cellular genes (51). To determine whether the differences in accumulation of β-actin and β-tubulin mRNAs could be the result of ICP27 affecting transcription of these genes, we performed nuclear runoff transcription assays (Table 1). Results from three separate experiments showed that while infection with KOS 1.1 did in fact inhibit transcription of β-actin three- to fivefold and of β-tubulin two- to fivefold, the reductions in transcription initiation in *ts*LG4-infected cells in which ICP27 is defective were the same. Therefore, the differences in accumulation levels of host mRNA between wild-type-infected cells and ICP27 mutant-infected cells were not due to transcription effects of ICP27. Instead, these differences occurred posttranscriptionally.

ICP27 does not appear to alter the stability of β-actin mRNA. Another possibility that accounts for the reduced accumulation of cellular mRNAs in KOS 1.1-infected cells could be alterations in the stability of cellular RNAs caused by ICP27. Mosca et al. (31) reported that an HSV-1 product increased the stability of otherwise unstable interferon mRNA. Our findings would require ICP27, or some product regulated by ICP27, to destabilize cellular mRNAs. To test this possibility, we measured the turnover of β-actin mRNA in cells infected either with KOS 1.1 or with the ICP27 mutant viruses

TABLE 1. Transcription of cellular genes as measured by nuclear runoff assays with KOS 1.1-infected cells and *ts*LG4-infected cells

mRNA[a]	Transcription rate[b]					
	Expt 1		Expt 2		Expt 3	
	KOS	*ts*LG4	KOS	*ts*LG4	KOS	*ts*LG4
β-Actin	0.21	0.3	0.35	0.29	0.19	0.17
β-Tubulin	0.57	0.79	0.22	0.31	0.29	0.26

[a] RNA probes were prepared and bound to GeneScreen filters as described in Materials and Methods.

[b] Nuclei were isolated from Vero cells which were mock infected or infected with HSV-1 KOS 1.1 (KOS) or *ts*LG4 at 5 h (experiments 1 and 2) or 7 h (experiment 3) after infection. Transcription from preinitiated transcription complexes was continued for 30 min at 27°C in the presence of [^{32}P]CTP. Hybridized filters were counted by liquid scintillation counting after autoradiography of the slot blots. Values are presented as relative transcription rates, in which the value for mock-infected cells in each experiment was set equal to 1.0 and values for KOS- and *ts*LG4-infected cells were calculated relative to this value.

*ts*LG4 and 27-LacZ, in which the ICP27 gene has been inactivated by the insertion of *lacZ* (52). Infected cells were incubated for 4 h to allow expression of ICP27, and then actinomycin D was added to inhibit further transcription. Total cellular RNA was isolated at the time of actinomycin D addition and 2 and 4 h later. The amount of β-actin mRNA detected in KOS 1.1-infected cells at the time of actinomycin D addition was significantly less than those detected in 27-LacZ- or *ts*LG4-infected cells, again demonstrating the difference in accumulation of host mRNA when ICP27 is functional (Fig. 3A). The fractions of mRNA remaining 2 and 4 h after actinomycin D addition were not substantially greater for samples from 27-LacZ- or *ts*LG4-infected cells than they were for samples from KOS 1.1-infected cells; however, 1.7 times more β-actin mRNA was seen at 2 h after actinomycin D addition in the 27-LacZ sample. This could reflect a difference in the stability of β-actin mRNA in KOS 1.1-infected cells, or it could reflect differences in the processing of β-actin pre-mRNA. To determine if the stabilities of spliced β-actin mRNA in wild-type KOS 1.1-infected cells and in 27-LacZ-infected cells were equivalent, a second approach in which cytoplasmic rather than total RNA was isolated at 4 h after infection was used. Again, actinomycin D was added at this time and cytoplasmic RNA was isolated 2, 4, and 6 h after addition of the inhibitor. As can be seen in Fig. 3B, the decay of β-actin mRNA in the cytoplasm of KOS 1.1-infected cells was similar to that of 27-LacZ-infected cells. Densitometer scanning of the autoradiographs showed that about 35% of β-actin mRNA remained 2 h after addition of actinomycin D to the sample from KOS 1.1-infected cells, whereas about 33% of β-actin mRNA remained in the sample from 27-LacZ-infected cells. These data indicate that the cytoplasmic stability of β-actin mRNA appears to be unaffected by the expression of ICP27, although accumulation of the spliced product in KOS 1.1-infected cells is decreased. These data suggest that conversion of pre-mRNA to spliced product and/or the nuclear stability of pre-mRNA may be affected by ICP27.

Functional ICP27 is required for the decrease in spliced mRNA accumulation. We next sought to determine whether decreased accumulation of cellular mRNAs was due to the activity of the ICP27 protein directly or indirectly through the action of some viral product which requires ICP27 for its expression. This question was resolved by two approaches. To address whether ICP27 alone would be sufficient to cause decreased accumulation of spliced mRNAs, a transfection

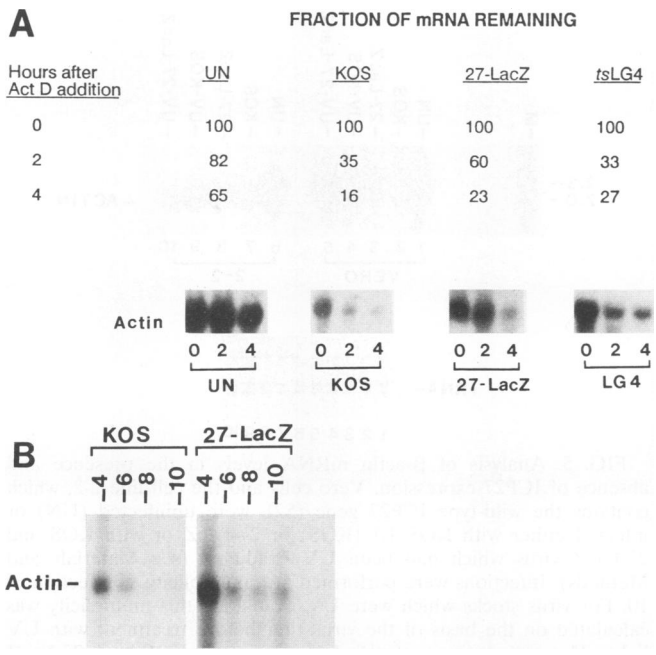

FIG. 3. Decay of β-actin mRNA. (A) Vero cells were left uninfected (UN) or were infected with KOS 1.1 (KOS), 27-LacZ, or *ts*LG4 at a multiplicity of infection of 10 at 39.5°C. Four hours after infection, RNA was isolated from one set of cultures (zero time) for each infection, and actinomycin D (10 μg/ml) (Act D) was added to the remaining cultures. RNA was isolated 2 and 4 h after the addition of Act D. Equal amounts of total RNA (20 μg) were fractionated in agarose for each sample. Northern hybridization analysis was performed as described in Materials and Methods with a ^{32}P-labeled β-actin antisense RNA probe. Several exposures of each Northern hybridization were made, and different exposures were scanned for each condition by a laser densitometer to quantify the levels of RNA and to ensure that the readings were in linear range for each set of samples. The fraction of mRNA remaining was calculated on the basis of the amount of β-actin mRNA seen for each sample at the time of addition of Act D (zero time). (B) Vero cells were infected with KOS or 27-LacZ at a multiplicity of infection of 10 for 4 h. At this time, cytoplasmic RNA was isolated from one set of cultures while Act D was added to the remaining cultures. Cytoplasmic RNA was isolated 2, 4, and 6 h after addition of Act D. Equivalent amounts of RNA (10 μg) were fractionated in agarose, and Northern blots were hybridized with an RNA probe specific for β-actin RNA. The autoradiograph was scanned with a laser densitometer to quantify the results.

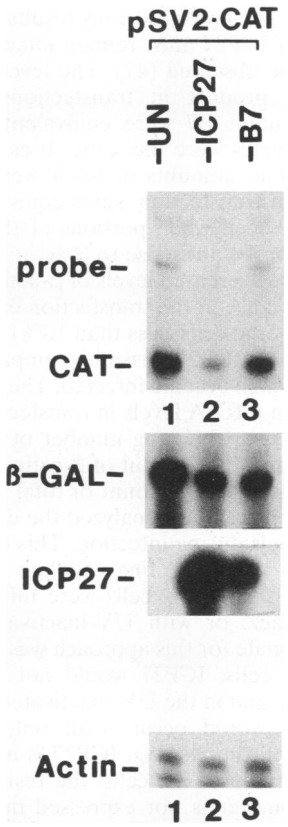

FIG. 4. ICP27, in the absence of other viral products, can decrease mRNA accumulation from a target gene containing an intron. Transfections were performed with the target plasmid pSV2-CAT (16), which contains the SV40 small t antigen intron 3′ to the CAT coding sequences. Cells were either transfected with pSV2-CAT alone (lanes 1) or were cotransfected with a plasmid containing the wild-type ICP27 gene (pSG130B/S) (lanes 2) or with a plasmid containing the ICP27 insertion mutant B7 (lanes 3). A plasmid containing the *lacZ* gene under the control of the cytomegalovirus promoter was included in the transfections as a control for transfection efficiency. Total RNA was isolated 36 h after transfection, and RNase protection assays were performed. A CAT-specific antisense RNA probe which spans 250 nucleotides at the 5′ end of CAT mRNA (47) was used in the protections shown in the uppermost panel. A β-galactosidase-specific (β-GAL) RNA probe which spans 250 nucleotides at the 5′ end of β-GAL RNA was used in the protections shown in the panel beneath the uppermost panel. An ICP27-specific RNA probe was used in the middle panel. To determine if equivalent amounts of RNA were recovered and analyzed, a fraction of each sample representing 1/10 of the amount of RNA used in the upper panels was hybridized with a β-actin RNA probe. The actin protection assay is shown in the bottom panel.

experiment was performed. We had previously shown that in the presence of ICP27, spliced target mRNA levels were decreased 5- to 10-fold in transfections with target genes containing a 5′ or 3′ intron (47). In those experiments, plasmids expressing the HSV-1 transactivators ICP4 and ICP0 were also present. To avoid including any HSV-1 gene products other than ICP27, transfections were performed with the target plasmid pSV2-CAT, which contains the strong SV40 early promoter and enhancer and is abundantly expressed. In addition, this plasmid contains the small t antigen intron derived from SV40 (16). Wild-type ICP27 was expressed from a plasmid termed pCMV-27, in which the ICP27 gene is placed under the control of the strong immediate-early promoter and enhancer from human cytomegalovirus (2). The mutant B7, which contains an insertion in the repressor region of ICP27 (17), was also cloned under the cytomegalovirus immediate-early promoter. As a control for transfection efficiencies, a plasmid which encodes the *lacZ* gene encoding β-galactosidase was also included. The *lacZ* gene was also placed under the control of the cytomegalovirus promoter. RNase protection experiments were performed with total RNA samples isolated from transfections with pSV2-CAT alone (Fig. 4, lane 1) or in the presence of wild-type ICP27 (lane 2) or mutant B7 (lane 3). Protections with a probe antisense to the 5′ region of CAT mRNA showed that about six times more CAT RNA was present in cells transfected with pSV2-CAT alone than was found in the presence of ICP27 (Fig. 4). This result indicates that ICP27 alone is sufficient to decrease accumulation of CAT RNA. About four times more CAT RNA was found in the transfection with B7 than in the transfection with wild-type

ICP27, which correlates with previous results showing that the repressor region of ICP27 must remain intact for its effect on spliced RNAs to be observed (47). The levels of the β-galactosidase-protected product in transfections with wild-type ICP27 and the mutant B7 were equivalent, indicating that transfection efficiencies were the same. It can also be seen in Fig. 4 that equivalent amounts of RNA were recovered and analyzed from each transfection, since equivalent amounts of β-actin RNA were seen when portions of the RNA samples hybridized with a probe antisense to β-actin RNA. This result is different from the decreased levels of β-actin RNA observed in infected cells because in the transfection experiment, only a small percentage of the cells (less than 10%) were successfully transfected with the ICP27 plasmid compared with nearly 100% of the cells which became infected. Therefore, the effects of ICP27 on β-actin mRNA levels in transfection assays would be masked by the overwhelming number of cells which were not transfected. Thus, the amount of β-actin RNA analyzed is an accurate measure of the amount of total RNA analyzed.

In the second approach, we analyzed the effect of ICP27 on cellular mRNA levels during infection. This experiment made use of the ICP27-complementing cell line 2-2 (52). In this experiment, Vero cells or 2-2 cells were infected either with KOS 1.1 or 27-LacZ or with UV-inactivated KOS 1.1 or 27-LacZ. The rationale for this approach was as follows. In the infection of Vero cells, ICP27 would not be expressed by 27-LacZ infections, and in the UV-inactivated virus infections, no viral synthesis would occur, with only those products present in the virion functioning. ICP27 is not present in the virion (62). In the case of 2-2 cells, the resident ICP27 gene under its own promoter is not expressed during the normal growth of cells, but upon infection with HSV-1, VP16 in the virion induces expression of the cellular ICP27 (3, 25). Therefore, infection with 27-LacZ would yield a wild-type infection, whereas infection with UV-inactivated virus would result in the expression of ICP27 as the only viral product except for virion proteins. As seen in Fig. 5, β-actin mRNA levels in KOS 1.1-infected Vero cells were reduced about fivefold compared with those in Vero cells infected with 27-LacZ or UV-inactivated virus. Levels in these three infections, although higher than in KOS 1.1-infected Vero cells, were still about twofold lower than in the uninfected control. This is due to the action of the *vhs* product. In contrast, β-actin mRNA levels were reduced to the same extent in 2-2 cells infected with 27-LacZ or UV-inactivated virus as they were when 2-2 cells were infected with KOS 1.1. To ensure that equivalent amounts of total RNA were analyzed, a portion of each sample was fractionated in agarose and stained with ethidium bromide to visualize the 28S and 18S rRNAs. A negative of the stained gel, shown at the bottom of Fig. 5, was scanned by laser densitometry. The maximum difference in the amounts of RNA analyzed by Northern hybridization was about 1.3-fold as seen in Fig. 5, lanes 9 and 10. Therefore, equivalent amounts of RNA were analyzed for each sample, and the differences cannot be attributed to sample loading variations.

The results presented above suggest that expression of ICP27 in the 2-2 cell line was responsible for the decreased accumulation of β-actin mRNA which was seen. This is because the only HSV-1 product expected to be expressed, in addition to the virion proteins, in 2-2 cells infected with UV-inactivated virus was ICP27. To confirm that this was the case, immunofluorescent staining was performed on cells infected with KOS 1.1 or UV-inactivated KOS 1.1. Monoclonal antibodies to ICP4, ICP0, ICP27, and ICP8 were used. Infection of 2-2 cells with KOS 1.1 resulted in characteristic nuclear staining of ICP0 (Fig. 6E), ICP4 (panel G), and ICP8 (panel I).

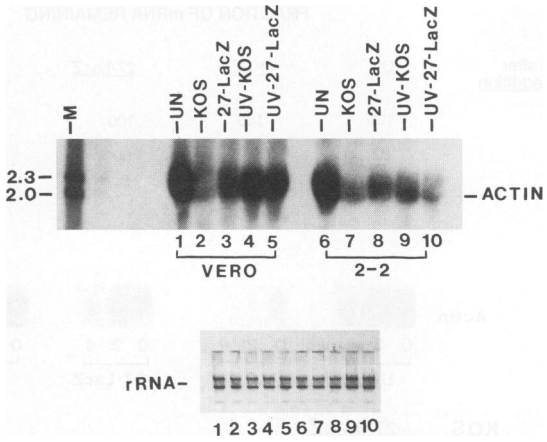

FIG. 5. Analysis of β-actin mRNA levels in the presence and absence of ICP27 expression. Vero cells and the cell line 2-2, which contains the wild-type ICP27 gene (52), were uninfected (UN) or infected either with KOS 1.1 (KOS) or 27-LacZ or with KOS and 27-LacZ virus which had been UV irradiated (see Materials and Methods). Infections were performed at a multiplicity of infection of 10. For virus stocks which were UV irradiated, this multiplicity was calculated on the basis of the virus titer before treatment with UV light. The actual titers of the UV-inactivated KOS and 27-LacZ samples were reduced to <100 PFU/ml after irradiation. RNA was isolated 5 h after infection. The RNA blot was hybridized with a ^{32}P-labeled β-actin antisense RNA probe. To ensure that equivalent amounts of RNA were analyzed, portions of each sample were fractionated in agarose and stained with ethidium bromide to visualize the 28S and 18S rRNAs. The gel was photographed with positive/negative film. The negative, shown in the bottom panel, was scanned by laser densitometry to quantify the amounts of rRNA. Lane M, the positions of 2.3- and 2.0-kb *Hin*dIII fragments from λ bacteriophage DNA which were ^{32}P labeled and fractionated with the RNA samples to serve as size markers.

Similarly, defined nuclear staining of ICP27 was seen with Vero cells infected with KOS 1.1 (Fig. 6A) and 2-2 cells infected with 27-LacZ (panel C). In contrast, infection of 2-2 cells with UV-inactivated KOS 1.1 showed no staining above background level for ICP0 (Fig. 6F), ICP4 (panel H), and ICP8 (panel J). Infection of Vero cells with UV-inactivated KOS 1.1 showed no staining with antibody to ICP27 (Fig. 6B). However, 2-2 cells infected with UV-inactivated KOS 1.1 did show nuclear staining of ICP27 (Fig. 6D). These results confirm that ICP27 was expressed in 2-2 cells infected with UV-inactivated virus. Further, they show that ICP27 was the only de novo viral gene product expressed, because expression of ICP4, ICP0, and ICP8 was not detected. Because ICP4 is required for HSV-1 early and late gene expression, it is unlikely that other early gene products not monitored in this study were expressed. These results show that since the virion proteins alone were not sufficient to cause the additional effect on β-actin mRNA levels seen in Vero cell infections with UV-inactivated virus, ICP27 expression appears to be sufficient for decreased accumulation.

The repressor region of ICP27 must be intact for the effects on cellular mRNA levels. We previously showed that the region defined as the activator region of ICP27 (17) was not required for the down regulation of spliced products in transfection assays, whereas the repressor region was required to be intact for this effect (17, 47). The mutation in *ts*LG4 was shown to map in the repressor region of ICP27 (54), and as shown here, this viral mutant was not able to reduce the accumulation of

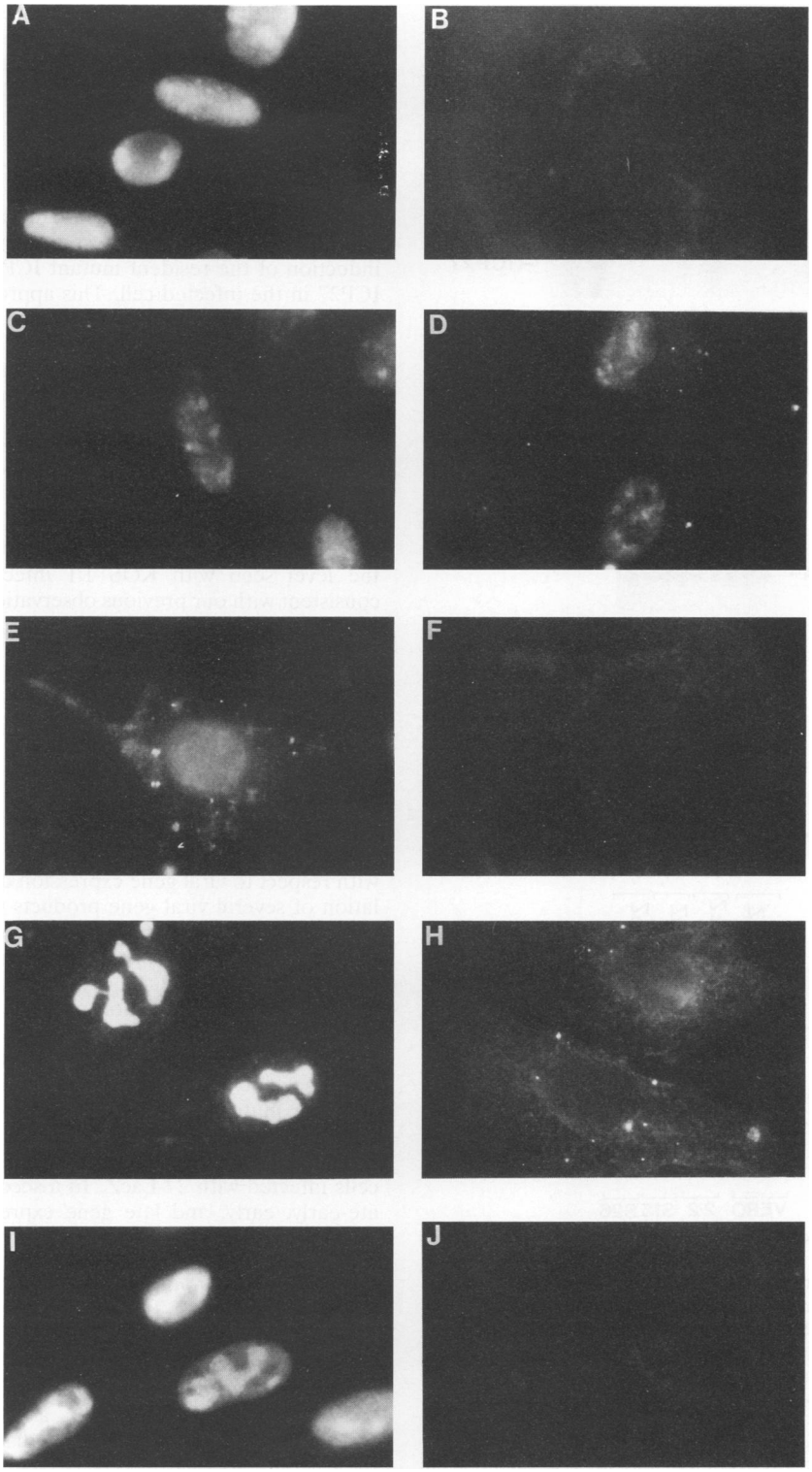

FIG. 6. Immunofluorescent staining of cells infected with UV-inactivated virus. Vero cells (A and B) and 2-2 cells (C through J) were infected with KOS 1.1 (A, E, G, and I), 27-LacZ (C), or UV-inactivated KOS1.1 (B, D, F, H, and J). Five hours after infection, cells were fixed and stained with monoclonal antibodies to ICP27 (A, B, C, and D), ICP0 (E and F), ICP4 (G and H), or ICP8 (I and J).

cellular mRNA. To determine what effect other activator and repressor mutants would have on cellular mRNAs, we analyzed the effects of mutant S23, an ICP27 activator mutant which has an insertion of 2 amino acids at residue 383 (17, 54), and mutant S1B, which has a 4-amino acid insertion at residue 406 (17, 54). This mutation, previously defined as an activator mutation, maps near the border of what we defined as the repressor region (17). In previous studies with these mutants,

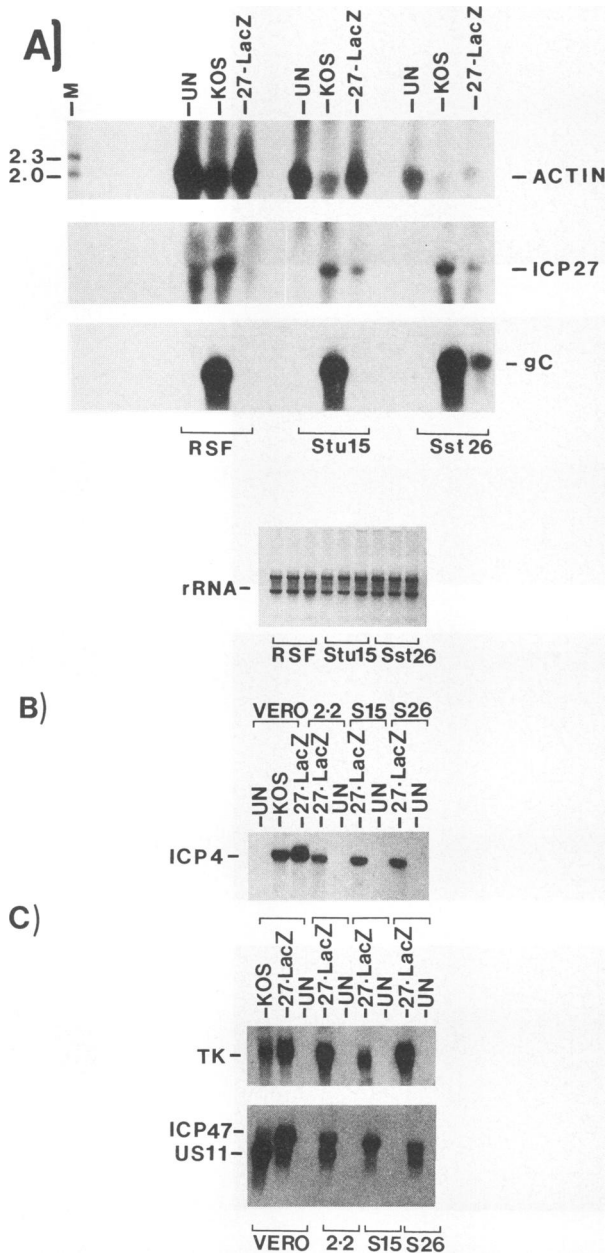

FIG. 7. Analysis of β-actin mRNA levels in cell lines expressing ICP27 mutants. (A) Rabbit skin fibroblast cells and the cell lines *Stu15*, which contains the ICP27 mutant S1B, and *Sst26*, which contains the ICP27 mutant S23 (54), were uninfected (UN) or infected with KOS 1.1 (KOS) or 27-LacZ. RNA was isolated 5 h after infection, and samples (20 μg) were fractionated in agarose and transferred to GeneScreen. To ensure equal loading of RNA samples, portions of each sample were fractionated in agarose and stained with ethidium bromide. The stained gel was photographed, and the negative, shown at the bottom, was scanned by laser densitometry to quantify the amounts of RNA. The blot was hybridized with a ^{32}P-labeled β-actin antisense RNA probe. Following autoradiography, the hybridized probe was stripped from the blot, which was then rehybridized with an antisense RNA probe specific for ICP27 (middle panel). The hybridized probe was again stripped from the blot, and the RNA was rehybridized with an antisense RNA probe specific for gC mRNA (lower panel). Size markers are shown in the upper panel (lane M) and are as described in the legend to Fig. 5. (B) Vero, 2-2, *Stu15*, and *Sst26* cells either were left uninfected or were infected with KOS or 27-LacZ. Total RNA was isolated at 5 h after infection in the Northern hybridization shown in panel B and 9 h after infection in the hybridizations shown in panel C. ^{32}P-labeled DNA probes specific for ICP4 and TK and a probe which hybridizes to both US11 and ICP47 were used to detect viral mRNAs.

we showed that S23 and S1B expressed proteins which were *trans* dominant to wild-type ICP27 and interfered with virus replication, most notably at low multiplicities of infection (54). Consequently, we have been unable to introduce these mutations into the viral genome by recombination. Instead, we have analyzed the mutant phenotypes by using cell lines stably transformed with the mutant genes. These cell lines, designated *Sst26*, which contains mutant S23, and *Stu15*, which contains mutant S1B, were isolated and characterized previously (54). Infection of these cells with 27-LacZ results in induction of the resident mutant ICP27 as the only source of ICP27 in the infected cell. This approach is similar to that of Gao and Knipe (13), who studied the effects of a *trans* dominant ICP8 protein during viral infection by using a cell line which expressed the *trans* dominant mutant. In the following experiments, infection of each mutant cell line with KOS 1.1 was performed at high multiplicity (multiplicity of infection of 10), at which the *trans* dominant effect is not seen (54), to directly compare the effects of the wild-type protein and the mutant. Infection of the *Sst26* cell line with 27-LacZ resulted in greatly reduced levels of β-actin mRNA detected relative to those of uninfected control cells, and the level was similar to the level seen with KOS 1.1 infection (Fig. 7A). This is consistent with our previous observation that activator mutants decreased expression from target genes containing introns as efficiently as the wild type (17, 47). By contrast, the level of β-actin mRNA in 27-LacZ-infected *Stu15* cells was substantially higher than in KOS 1.1-infected *Stu15* cells (Fig. 7A). This result indicates that the mutation in *Stu15* inactivates the effect which ICP27 has in decreasing accumulation of cellular mRNAs. In this respect, the *Stu15* mutant behaved like the previously defined repressor mutants, which decreased mRNA levels from spliced target genes.

To further characterize the phenotypes of these mutants with respect to viral gene expression during infection, accumulation of several viral gene products was also analyzed. Some viral ICP27 mutants have been found to overexpress some immediate-early and early gene products and to underexpress late gene products (27, 40, 44). The blot in Fig. 7A was stripped of the β-actin hybridization probe and was rehybridized first with a probe specific for ICP27 RNA to confirm expression in *Stu15* and *Sst26* cells and, after subsequent stripping, with a probe specific for the late gene product gC. As expected of an activator mutant, gC mRNA levels were about 30-fold lower during infection of the *Sst26* cell line with 27-LacZ than with KOS 1.1. In addition, gC mRNA was not detectable in *Stu15* cells infected with 27-LacZ. In a second experiment, immediate-early, early, and late gene expression was monitored in *Stu15* and *Sst26* cells compared with those of wild-type KOS 1.1 infection and 27-LacZ infection in Vero and 2-2 cells (Fig. 7B). The immediate-early gene product ICP4 did not appear to be overexpressed in either *Stu15* or *Sst26* cells compared with 2-2 cells infected with 27-LacZ or Vero cells infected with 27-LacZ or KOS 1.1 (Fig. 7B). However, TK mRNA levels were somewhat more abundant in *Sst26* than in *Stu15* cells. In the bottom panel of Fig. 7C, a probe which overlaps the coterminal mRNAs for the immediate-early gene ICP47 and the true late gene US11 was used. In the 27-LacZ infection of Vero and *Stu15* cells, only the immediate-early product was

found. In infections of 2-2 and *Sst26* cells, comparable amounts of ICP47 and US11 mRNA were seen. It was surprising that more US11 expression than gC expression was detected in the *Sst26* cell line. This result suggests that this mutation may render the ICP27 protein partially functional in its ability to induce late gene expression during infection. On the other hand, the mutation in *Stu15* results not only in the inability of the mutant protein to decrease cellular mRNA levels as seen in Fig. 7A but also in the inability to induce viral late gene expression.

Precursor RNA can be detected in cells infected with wild-type HSV-1. While we have shown that ICP27 causes decreased accumulation of some cellular mRNAs which was not the result of a depression of transcription or reduced stability of the mature mRNAs, these findings do not directly demonstrate that splicing is affected. In a related study, Schroder et al. (49) analyzed the processing of β-tubulin heterogeneous nuclear RNA in HSV-1-infected cells and found that processing of β-tubulin heterogeneous nuclear RNA was reduced by 2 h after infection and inhibited almost completely by 5 h. This resulted in accumulation of unspliced β-tubulin pre-mRNA. We have been unable to detect precursor RNA for the cellular transcripts we have analyzed. This is probably because unspliced RNA is rapidly degraded in the nucleus, the cellular mRNAs we analyzed are not transcribed to high levels normally, and their transcription rate is further reduced by HSV-1 infection (Table 1), making it even more difficult to detect precursors. In addition, the probes we used were derived from cDNAs, not genomic clones. Therefore, we chose to focus on a viral product which is spliced and abundantly transcribed early during infection, namely ICP0. The ICP0 gene is 3,587 bp long and composed of three exons (35). Experiments were performed with an RNA probe which spanned an intron-exon junction so that both precursor and spliced product would be detected (see Materials and Methods). In the first experiment, total RNA was isolated from Vero and 2-2 cells infected with KOS 1.1 or 27-LacZ. In addition, infections were performed with a virus termed 27R, which was constructed by marker rescue of the chimeric ICP27-*lacZ* gene in the 27-LacZ virus with a DNA fragment spanning the wild-type ICP27 gene. Infections were performed with the rescued virus 27R to further confirm that ICP27 was responsible for the effects observed on spliced mRNAs. Equivalent amounts of total RNA from each sample were analyzed by Northern blot hybridization. That this was the case can be seen in the lowest panel of Fig. 8, where the blot was hybridized with a probe specific for 28S rRNA. The uppermost panel of Fig. 8 shows that levels of β-actin mRNA were reduced during infection of Vero and 2-2 cells with KOS 1.1 and the rescued virus 27R. As was seen previously, β-actin mRNA levels were higher in Vero cells infected with 27-LacZ (Fig. 8, top panel), in which ICP27 was not expressed, but were equivalent to those of KOS 1.1 and 27R infections in 2-2 cells, in which ICP27 was expressed by the cell line. The blot in the uppermost panel was then stripped of the hybridization probe and was rehybridized with an ICP0 antisense RNA probe which spans the junction of intron 1 and exon 2 (see Materials and Methods). In this case, ICP0 precursor mRNA was clearly detectable in KOS 1.1 and 27R infections of Vero cells and in all of the infections of 2-2 cells. In fact, the product-to-precursor ratio was around 6:1. Smaller amounts of ICP0 mRNA were seen in the 27-LacZ infection of Vero cells than in KOS 1.1 or 27R infections; however, even upon longer exposure of the autoradiogram, as seen in the panel immediately below the one mentioned above, ICP0 pre-mRNA was not detected in the RNA sample from 27-LacZ-infected Vero cells. In contrast, it was detected in equivalent amounts in 2-2 cells infected with 27-LacZ, KOS 1.1, and 27R. This indicates that ICP27 is required not only for reduction in spliced mRNA levels but also for accumulation of precursor mRNA.

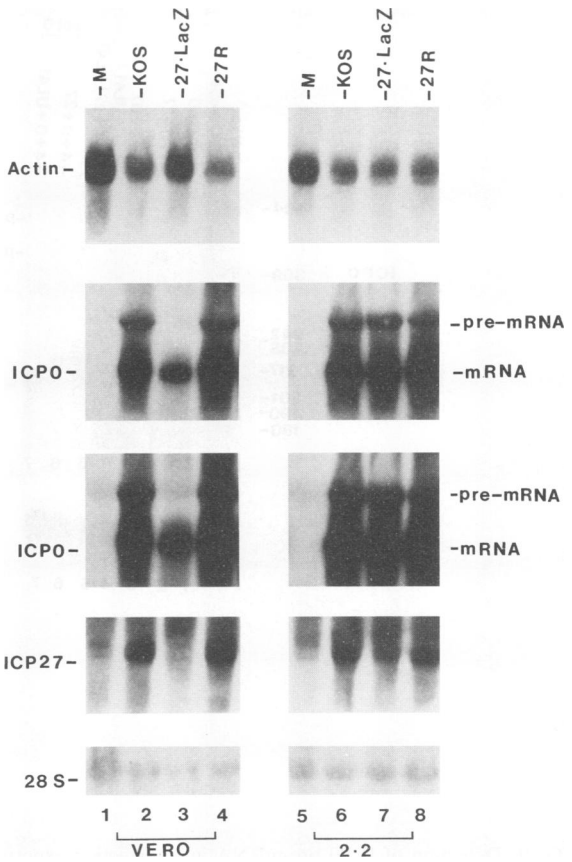

FIG. 8. Detection of ICP0 pre-mRNA during infection. Vero and 2-2 cells were mock infected (lanes M) or infected with KOS 1.1 (KOS), 27-LacZ, or the rescued virus 27R, in which the wild-type ICP27 gene was restored in 27-LacZ virus by marker rescue. Total RNA was isolated 5 h after infection. Twenty-microgram samples of RNA were loaded onto the glyoxal gels. That equivalent amounts of total RNA were loaded can be seen in the hybridization of the Northern blot with a 28S rRNA probe as shown at the bottom. The RNA blot was first hybridized with an RNA probe specific for β-actin mRNA (uppermost panel). The hybridized probe was then stripped, and the blot was rehybridized with an antisense RNA probe which hybridizes to a portion of intron 1 and exon 2 in ICP0 RNA. A darker exposure of each autoradiogram is also shown immediately below. After hybridization to ICP0 mRNA, the probe was again stripped and the RNA blot was hybridized to an RNA probe specific for ICP27 RNA. Finally, the blot was stripped of the ICP27 hybridization probe and was rehybridized with a probe specific for 28S rRNA.

Accumulation of ICP0 precursor mRNA in the presence of ICP27 but not in its absence suggests that ICP27 exerts its influence on mRNA at the level of splicing. To explore this possibility further and to clearly distinguish the effect of ICP27 on mRNA levels compared with that of the UL41 *vhs* product, we performed a transfection experiment with plasmids expressing ICP27 or UL41. We measured the amounts of RNA recovered from two spliced RNA species and two unspliced RNA species by RNase protection assay. A target plasmid termed pTK-CAT-5′S-SVLPA (47), which is under the control of the HSV-1 TK promoter and contains alternative splice sites

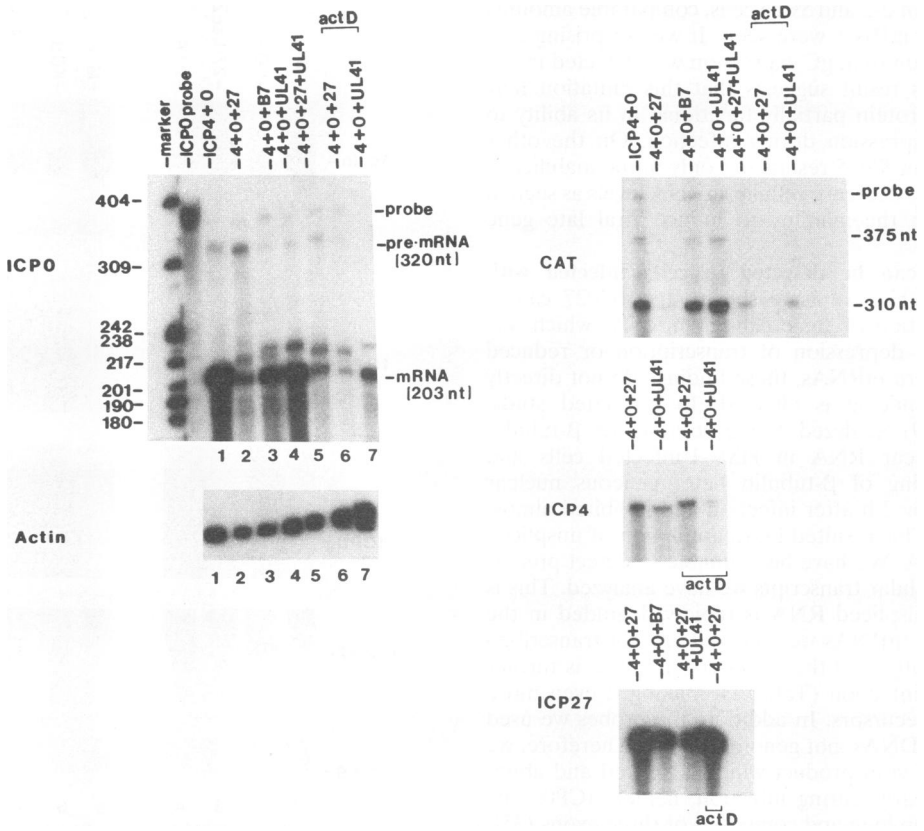

FIG. 9. Detection of ICP0 pre-mRNA in transfection experiments in the presence and absence of ICP27. Rabbit skin fibroblast cells were cotransfected with the plasmid pTK-CAT-5′S-SVLPA and with plasmids pSG28K/B, which encodes ICP4, and pRS-1, which encodes ICP0. Plasmid pSG130B/S, encoding ICP27, and plasmid pB7, which encodes an ICP27 insertion mutant, were added to some of the transfections as indicated. A plasmid termed pUL41, which encodes the HSV-1 UL41 gene, was included in some of the transfections as indicated. RNA was isolated at 36 h after transfection (lanes 1 through 5). Lanes 6 and 7, actinomycin D (actD) (10 μg/ml) was added to the transfected cells at 36 h and RNA was harvested 6 h later. An antisense RNA probe which encompasses part of intron 1 and part of exon 2 of ICP0 was used to detect ICP0 pre-mRNA and mRNA. An antisense probe which encompasses 250 nucleotides (nt) in the CAT coding sequence and the 5′ intron which contains alternative splice acceptor sites (47) was used to detect alternatively spliced CAT mRNAs. Antisense RNA probes were also used to detect ICP4 and ICP27 mRNA as described in Materials and Methods. A portion of each sample representing 1/20 of the amount of RNA analyzed in the other protection experiments was hybridized with a probe specific for β-actin mRNA to ensure that equivalent amounts of RNA were recovered and analyzed from each transfection. Lane marker, ^{32}P-labeled *Hin*fI-digested pBR322 DNA.

5′ to the CAT gene, was cotransfected with plasmids expressing ICP4 and ICP0. These transactivators were added to induce expression of the TK promoter and to induce expression of the plasmid encoding UL41, which was added to some of the transfections. As can be seen in the upper right panel of Fig. 9, the amounts of the 310-nucleotide spliced CAT mRNA product and the alternatively spliced 375-nucleotide CAT mRNA product were reduced more than fivefold in transfections which included ICP27. The UL41 product did not appear to affect accumulation of the spliced CAT mRNA in the sample isolated 36 h after transfection. It is possible that UL41 levels were not high enough to see the destabilizing effect while transcription was occurring. The UL41 product was found at much lower levels than ICP4 and ICP27 in the RNase protection assay (data not shown). However, in a parallel sample in which actinomycin D was added to the transfected cells at 36 h and RNA was isolated 6 h later, there was about an eightfold decrease in the amount of CAT mRNA found in the presence of UL41. Because CAT mRNA was barely detectable in the presence of ICP27, it was not possible to determine the effects on the stability of the spliced product when actinomycin D was similarly added to a transfection which included ICP27. ICP0 was present in all of the transfections, and therefore, the level of product to precursor was determined with the antisense RNA probe used in the Northern hybridization shown in Fig. 8. The full-length probe, which encompasses part of intron 1 and exon 2, is 375 nucleotides, including about 50 nucleotides of transcription vector sequences. The fully protected pre-mRNA product was expected to be around 320 nucleotides, and the fully spliced mRNA product was 203 nucleotides. In addition, a band of around 220 nucleotides was seen. This represents a product which likely resulted from incomplete digestion with RNase. Incubation at 45°C during RNase digestion eliminated this product (data not shown); thus, it may result from the formation of secondary structures. In calculating amounts of RNA, this product was included with the fully processed mRNA. In the transfection in which ICP27 was present, there was about 2.5 to 7 times less ICP0 mRNA detected (Fig. 9, upper left panel, lane 2) than in transfections in which ICP27 was not present (lanes 1 and 4) or in which the repressor mutant B7 was used (lane 3). However, a higher ratio of precursor to product was seen in the presence of ICP27. There was about 1/4 as much pre-mRNA as mRNA in the presence of wild-type ICP27, whereas the ratio was 1/10 to 1/20

as much in the absence of ICP27 or with the mutant B7. This result indicates that ICP27 causes an accumulation of pre-mRNA which results in a concomitant decrease in spliced product. In contrast, UL41 has its effect on the level of the spliced product, as has been shown previously (37, 48, 55). Thus, the levels of ICP4 mRNA and ICP27 mRNA, two species which are not spliced, in transfections with ICP27 before the addition of actinomycin D and 6 h after addition were similar, again illustrating that ICP27 does not affect the stability of mRNAs (Fig. 9, middle and bottom panels on the right). The level of ICP4 was reduced after incubation with actinomycin D when UL41 was present, as was the level of CAT mRNA.

To monitor accumulation of ICP0 precursor mRNA during viral infection in wild-type- and ICP27 mutant-infected cells, RNase protection assays were performed on total RNA samples isolated 4 h after infection. The RNA probe used in the experiment was larger than the probe used earlier, and it included additional intron and exon sequences. The pre-mRNA was expected to be 435 nucleotides, and the fully spliced product was 253 nucleotides. The precursor product was detected in KOS 1.1-infected Vero and 2-2 cells but not in 27-LacZ-infected Vero cells (Fig. 10A). The darker exposure in the far right panel of Fig. 10A shows that precursor ICP0 RNA can be detected in 27-LacZ-infected 2-2 cells, in which ICP27 is expressed, but not in Vero cells even with overexposure of the autoradiograph. This result is consistent with the results presented in Fig. 8, and it indicates that ICP27 expression is required for accumulation of ICP0 pre-mRNA. ICP0 pre-mRNA was also detected by Northern blot analysis in KOS 1.1-infected Vero cells (Fig. 10C), in which both the precursor (3.8 kb) and spliced product (2.8 kb) were seen. Again, ICP0 pre-mRNA was not found in 27-LacZ-infected Vero cells (Fig. 10C).

Next, we analyzed ICP0 expression in the presence of ICP27 activator and repressor mutants to determine if detection of ICP0 pre-mRNA correlated with the effects seen with these mutants on accumulation of spliced cellular mRNAs. Infection of the activator mutant-containing cell line *Sst26* with 27-LacZ resulted in ICP0 pre-mRNA amounts similar to those seen in KOS 1.1-infected cells (Fig. 10B). However, there was significantly less ICP0 pre-mRNA detected in *ts*LG4-infected cells than in KOS 1.1-infected cells (Fig. 10B), in accord with this mutant's inability to reduce levels of spliced cellular mRNAs. These results suggest that reduction in cellular mRNA levels and accumulation of ICP0 pre-mRNA are related and are caused by an impairment in host cell splicing. These data further suggest that the repressor region of ICP27 must be intact for this effect on splicing to occur.

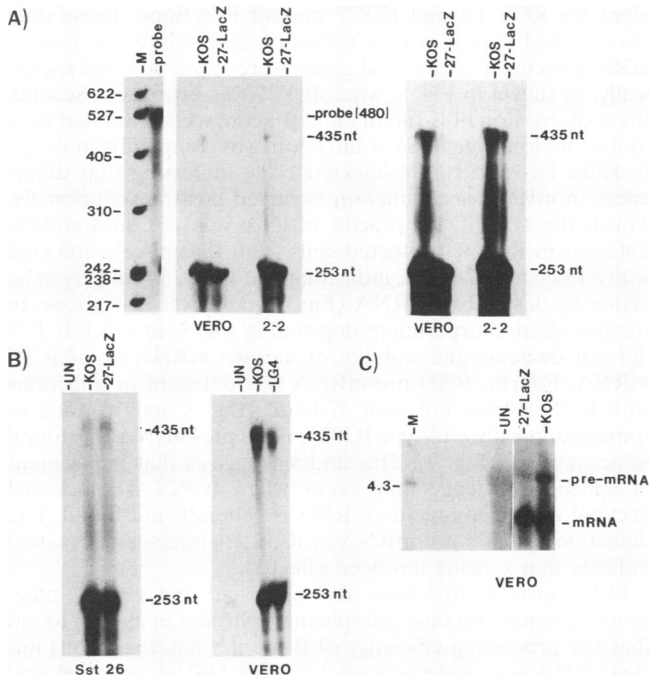

FIG. 10. Detection of ICP0 pre-mRNA during infection with wild-type HSV-1 and ICP27 mutants by RNase protection analysis. Vero and 2-2 cells were infected with KOS 1.1 (KOS) or 27-LacZ, and RNA was isolated 4 h after infection (A). RNase protections shown in panel A were performed with an antisense probe which spanned a portion of intron 1 and exon 2 of the ICP0 gene (see Materials and Methods). The ICP0 probe is shown along with size markers (lane M) which were obtained by end labeling *Hin*fI-digested pBR322 DNA. At the far right is a much longer exposure of the autoradiograph shown immediately to the left. Accumulation of ICP0 pre-mRNA in the presence of an ICP27 activator mutant (S23) and repressor mutant (*ts*LG4) was also analyzed (B). The cell line *Sst26*, which contains S23, and Vero cells were infected with KOS, 27-LacZ, or *ts*LG4 as indicated. RNase protections were performed on RNA isolated 4 h after infection. The pre-mRNA protected product is 435 nucleotides (nt) while the spliced protected product is 253 nt. ICP0 pre-mRNA was also detected by Northern blot hybridization (C). RNA samples from panel A were fractionated and transferred to a filter which was hybridized with the ICP0 antisense RNA probe used in the protection experiments. A 4.3-kb λ DNA size marker can also be seen (lane M). The λ DNA size markers were obtained by end labeling *Hin*dIII-digested λ DNA.

DISCUSSION

Inhibition of host protein synthesis by HSV-1 is a multistep process, with the primary phase involving mRNA degradation mediated by the *vhs* (UL41) component of the virion (7, 8, 10, 18, 22, 34, 37, 48, 55, 57, 58). A secondary stage of shutoff which appears to require de novo viral protein synthesis has also been described before (33). However, it is unclear at what level this secondary phase of shutoff occurs and what viral protein is involved. Studies on ICP27 *ts* mutants by Sacks et al. (44) showed that the shutoff of host protein synthesis was greatly reduced when ICP27 was defective. Furthermore, studies in our laboratory showed that spliced RNA products from target genes containing introns were found in smaller amounts in the presence of ICP27 (47). From these studies, we postulated that ICP27 could affect host cell splicing. If that was the case, ICP27 could contribute to the shutoff of host protein synthesis by reducing the level of spliced transcripts available for translation. In this work, we measured accumulation of three cellular mRNAs during infection and found that the levels of these mRNAs were significantly reduced when ICP27 was functional.

These data do not directly demonstrate an impairment of host splicing. However, we analyzed other control steps which could account for the differences seen and found that these steps are not likely to be involved. First, the reduced accumulation of β-tubulin and β-actin which occurred when ICP27 was functional was not the result of differences between the activity of the *vhs* protein present in ICP27 mutant virions and that of the wild type. This was demonstrated for *ts*LG4 by preventing viral transcription with actinomycin D, as shown in Fig. 2, and for 27-LacZ by the UV inactivation experiment shown in Fig. 5. In both instances, levels of cellular mRNAs, while lower than those found in uninfected cells, were equiv-

alent for KOS 1.1 and ICP27 mutant infections. These data also showed that a further reduction in β-tubulin or β-actin mRNA occurred when viral genes were expressed and specifically, as shown in Fig. 5, when ICP27 was expressed. Second, the transcription of β-tubulin and β-actin, while inhibited two- to fivefold following HSV-1 infection, was comparably reduced in KOS 1.1- and *ts*LG4-infected cells, indicating that differences in mRNA accumulation occurred posttranscriptionally. Third, the stability of β-actin mRNA was not substantially different in KOS 1.1-infected cells from that in cells infected with *ts*LG4 and 27-LacZ, indicating that ICP27 is unlikely to be acting by destabilizing RNA (Fig. 3). This was also shown in the transfection experiment depicted in Fig. 9, in which ICP27 did not decrease the stability of its own mRNA or of ICP4 mRNA. Fourth, ICP0 pre-mRNA was detected in infections with KOS 1.1 but not with 27-LacZ (Fig. 8 and 10), and in transfection with wild-type ICP27, ICP0 pre-mRNA was found to accumulate (Fig. 9). This finding suggests that impairment of splicing efficiency may occur when ICP27 is functional because unspliced precursor RNA is generally not found. The detection of ICP0 pre-mRNA in KOS 1.1-infected cells could indicate that splicing has been affected.

Other investigators have also suggested that HSV-1 infection interferes with host cell splicing. Schroder et al. (49) found that the processing efficiency of β-tubulin heterogeneous nuclear RNA was decreased by 2 h after HSV-1 infection and almost completely inhibited by 5 h after infection as determined by accumulation of β-tubulin pre-mRNA. We have not detected β-tubulin or β-actin precursor RNA in the Northern blot hybridization experiments shown here. However, the use of more sensitive RNase protection assays along with a probe which spans an intron-exon junction, instead of a cDNA probe, enabled us to detect ICP0 pre-mRNA. In fact, we found ICP0 pre-mRNA by Northern hybridization with this probe. With a completely different approach, Martin et al. (26) also found evidence for effects on host cell splicing following HSV-1 infection. These investigators analyzed the distribution of host cell small nuclear ribonucleoprotein (snRNP) antigens after infection with HSV-1. Redistribution from the normally speckled appearance of the snRNPs was seen. Namely, formation of condensed snRNP clusters was seen to occur, with these clusters moving to the periphery of the nucleus as infection proceeded. As a result of these findings, Martin et al. (26) speculated that splicing may be impaired by HSV-1 infection because these changes could adversely affect spliceosome assembly. However, no correlation was found between expression of a particular viral protein and reassortment of snRNPs. We also found redistribution of snRNPs and the non-snRNP splicing factor SC35 (12) following infection with HSV-1 KOS 1.1. Importantly, this relocalization did not occur in 27-LacZ infections, indicating that ICP27 is required for this effect (45a). In a similar study, Phelan et al. (36) found that ICP27 alone could alter the distribution of snRNPs when ICP27-expressing plasmids were transfected into cells. These data all point to the possibility that HSV-1 infection inhibits splicing and that ICP27 may facilitate inhibition. We have tested that hypothesis directly by performing in vitro splicing reactions with HeLa cell nuclear extracts. Extracts were made from uninfected HeLa cells or cells infected with KOS 1.1 or 27-LacZ. The efficiency of splicing a β-globin substrate RNA was reduced 5- to 10-fold in extracts from KOS 1.1-infected cells compared with those from uninfected or 27-LacZ-infected cells, providing direct evidence that HSV-1 infection impairs splicing and that ICP27 is required for this effect (17a).

In previous studies, we defined activator and repressor regions of ICP27 and showed that these regions correlated with different activities carried out by ICP27 (17). The activator region was required for ICP27 to increase polyadenylation at specific poly(A) sites (29, 47). The repressor region was required for the 5- to 10-fold decrease seen in the level of spliced transcripts in transfections with intron-containing target genes (47). Here, we have also attempted to correlate decreased accumulation of cellular mRNAs and the presence of ICP0 pre-mRNA with specific regions in the ICP27 protein by using defined mutants of ICP27. The activator mutant S23, expressed in the cell line *Sst26* (54), had a phenotype similar to wild-type ICP27 in that β-actin mRNA levels were reduced and ICP0 pre-mRNA was detected (Fig. 6 and 7). This was consistent with previous studies of this activator mutant which showed that CAT expression from target genes with introns was downregulated in transfections with S23 (17) or infections of *Sst26* cells (54). In contrast, the lesion in *ts*LG4 maps to the repressor region of ICP27 (54). In accord with the phenotype of mutants within this region, cellular mRNA levels were not reduced in *ts*LG4 infections and ICP0 pre-mRNA was detected in much lower abundance than in a KOS 1.1 infection (Fig. 1, 4, and 7). Interestingly, when a plasmid subclone containing the ICP27 gene from *ts*LG4 was used in transfection assays, a repression of CAT expression nearly equivalent to that of wild-type ICP27 was seen at both 34 and 39°C (17). This could be the result of the very large number of copies of the *ts*LG4 gene which enter the transfected cell compared with the infected cell (6). The resultant high level of expression of the partially functional *ts* protein thus could produce responses which are different from those seen during viral infection. This may also be the situation seen with the *Stu15* cell line, which harbors the S1B mutant. The S1B mutation maps at residue 406, which is at the boundary of the activator region, with the repressor region defined as extending from residues 434 to 508 (17). However, McMahan and Schaffer (30) described a second, weaker repressor region which extended to amino acid 407. In transfection experiments, S1B caused a downregulation in CAT activity, as is the case with other activator mutants, but here cellular mRNA levels were not reduced in the *Stu15* cell line. Again, the differences between transfection experiments and infection of a stably transformed cell line are likely related to the unnaturally high levels of expression of the S1B mutant during transfection compared with the level expressed during infection of cell line *Stu15*. The results with *ts*LG4 and the *Stu15* cell line indicate that the C-terminal region of the protein must remain intact for the effects observed on cellular mRNAs to occur. Further, the previously defined repressor region of the protein likely extends at least through amino acid 406. Our studies will now be focused on determining how ICP27 causes the effects we have seen on RNA processing and also on determining what other regions of the protein besides the repressor region are functionally important in causing these changes.

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