Herpes Simplex Virus Inhibits Host Cell Splicing, and Regulatory Protein ICP27 Is Required for This Effect

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While the majority of metazoan genes and those of the DNA viruses which infect them contain introns which require RNA splicing, herpes simplex virus type 1 (HSV-1) encodes relatively few spliced products. We previously showed that the HSV-1 immediate-early protein ICP27 decreased the levels of spliced target mRNAs in transfections and spliced cellular mRNAs during infection, suggesting that ICP27 may function in impairing host cell splicing. Here, we show that during infections with the wild type, but not in infections with an ICP27 viral mutant termed 27-LacZ, precursor RNA accumulated for a virus transcript which contained introns. Pre-mRNA accumulation in the nucleus was greater than that in the cytoplasm, indicating that splicing rather than transport was affected. Furthermore, splicing of a β -globin pre-mRNA substrate was inhibited in nuclear extracts from wild-type-infected cells but not in extracts from cells infected with 27-LacZ. The inhibitory activity in extracts from wild-type-infected cells was able to reduce the splicing efficiency of competent extracts in biochemical complementation assays. ICP27 appeared to be responsible for this decrease, because the splicing activity of an extract from cells infected with an ICP27 *ts* mutant was significantly reduced after incubation of the extract at the permissive temperature to allow renaturation of the conformationally defective ICP27 protein. These results strongly suggest that HSV-1 infection inhibits host cell splicing through the action of ICP27.

In eukaryotes, primary transcripts of protein-coding genes frequently contain introns which are removed during RNA maturation by splicing. Intronless genes are rare in metazoans, whereas genes with multiple introns are common (21). Similarly, many eukaryotic DNA viruses encode genes which are spliced singly or multiple times. However, the human pathogen herpes simplex virus (HSV) is unusual even among members of the *Herpesviridae* in that the majority of its transcripts are not spliced (65). Furthermore, there is evidence to suggest that HSV type 1 (HSV-1) infection may actually inhibit host cell splicing. Schroder et al. (55) found that the processing efficiency of β -tubulin heterogeneous nuclear RNA was reduced by 2 h after infection with HSV-1 and was inhibited almost completely by 5 h after infection, resulting in an accumulation of unspliced β -tubulin precursor RNA. Similarly, we have found a decrease in the level of spliced mRNAs for three cellular genes which could not be accounted for by differences in the transcription of these genes following HSV-1 infection or by differences in the stability of the spliced mRNAs (22).

Lytic infection with HSV results in the repression of most host protein synthesis (17, 24, 62, 63). This inhibition of host protein synthesis is a multiphase process caused initially by a structural component in the virion, termed the *vhs* function, which facilitates disaggregation of cellular polyribosomes and degradation of host mRNAs (16, 27, 28, 48). A delayed or secondary stage of shutoff reduces the remaining levels of host protein synthesis, and this stage requires viral gene expression (15, 39, 48, 60). It is unclear at what level this secondary stage of shutoff occurs; however, it is conceivable that it could occur at the level of splicing. That is, interference with efficient

splicing of host cell pre-mRNA would result in a decrease of spliced products for translation. The protein responsible for the secondary shutoff has not been identified. However, studies by Sacks et al. (52) of HSV-1 mutants defective in the immediate-early regulatory protein ICP27 showed that the shutoff of host protein synthesis was greatly reduced when ICP27 was defective. ICP27 (IE63 or UL54) is a 63-kDa nuclear phosphoprotein which is required for virus late gene expression and efficient DNA replication and appears to contribute to the shutoff of host protein synthesis (7, 35, 50, 52, 57). In transfection experiments, ICP27 has been shown to stimulate the expression of some target genes and to repress the expression of others (10, 23, 38, 49, 51, 56, 61). Interestingly, studies in our laboratory showed that the regulatory activity of ICP27 seen in transfection experiments occurred posttranscriptionally at the level of mRNA processing (54). Activation or repression of target gene expression was independent of target gene promoter sequences but instead depended on the presence of different mRNA processing signals. The activation function of ICP27 seen with some target genes correlated with different polyadenylation signals, whereas the repressor function correlated with the presence of introns in the target gene. These results led us to suggest that ICP27 could influence the complex processes of polyadenylation and splicing (54). With respect to the first effect, we hypothesized that the stimulatory effect which ICP27 had on the utilization of some poly(A) sites may be related to the late processing factor (LPF), whose activity in HSV-1-infected cells was identified by McLauchlan et al. (37). Using *in vitro* polyadenylation assays, these investigators found that LPF selectively increased processing at the poly(A) site of an HSV-1 late gene (UL38). In subsequent studies using nuclear extracts from cells infected with an ICP27 viral null mutant (57), it was shown that ICP27 is required for the increase in utilization of the UL38 poly(A) site (36). These data reinforce the hypothesis that ICP27

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affects posttranscriptional processing of RNA and that it is a component of the LPF-mediated effect.

The repressor activity of ICP27 was linked to the presence of introns in the target gene (54). Spliced target mRNAs were decreased 5- to 10-fold when ICP27 was present in the transfections. This result led us to suggest that ICP27 may impair splicing and thus contribute to the shutoff of host protein synthesis. As a first step to testing this hypothesis, we analyzed the levels of spliced cellular mRNAs during HSV-1 infection. We found that in infections with viral mutants defective in ICP27, at least three spliced host mRNAs accumulated to levels that were higher than levels seen with wild-type-infected cells. Unspliced precursor mRNA was found for a viral transcript in wild-type HSV-1 infections but not in infections lacking ICP27, further suggesting that host cell splicing may be impaired when ICP27 is functional (22).

Here, we have analyzed the consequences of the proposed impaired splicing efficiency on the nuclear and cytoplasmic accumulation of two viral transcripts which contain introns, an immediate-early gene product (ICP0) and a late gene product (UL15). We found that precursor RNA, while detectable in the cytoplasm, accumulated to a greater extent in the nucleus, suggesting that splicing and not transport was being affected. Further, we tested directly whether HSV-1 infection inhibits host cell splicing by performing *in vitro* splicing assays using nuclear extracts from cells infected with HSV-1. Splicing was inhibited in extracts from wild-type-infected cells but not in extracts from cells infected with an ICP27 null mutant. Furthermore, in biochemical complementation assays, the inhibition in splicing seen in HSV-1-infected cell extracts could be imparted to nuclear extracts which were competent in splicing. ICP27 appeared to be responsible for the decrease in splicing activity because when extracts containing a temperature-sensitive ICP27 protein were incubated at permissive temperature to restore activity, the splicing efficiency of the extract was significantly reduced. These results extend our previous findings on the posttranscriptional effects of ICP27 on RNA processing and strongly suggest that ICP27 is required for the impairment of splicing seen in HSV-1-infected cells.

MATERIALS AND METHODS

Recombinant plasmids. The plasmids pRS-1, which contains the HSV-1 ICP0 gene; pSG130B/S, which contains the ICP27 gene; and pS18, which contains an ICP27 insertion mutant, were described previously (23). The plasmid pDS16, which contains an ICP0 cDNA clone, was kindly provided by S. Silverstein (70). These plasmids were used in the transfection experiment shown in Fig. 1. Transfections were performed as described previously (23). Plasmid pSP64-H β 86, which was derived from the human β -globin gene, was kindly provided by M. Green (26). RNA splicing substrate was prepared from H β 86 linearized with *Bam*HI by SP6 *in vitro* transcription. T7 expression plasmids for *in vitro* transcription of antisense RNA probes specific for intron-exon junctions in the HSV-1 genes ICP0 and UL15 were constructed as follows. A 250-bp *Bam*HI-*Ava*I fragment from the ICP0 gene which spans a portion of intron 1 and exon 2 was cloned into the *Sma*I site of pGEM2 (Promega), after appropriate modification of the ends. The plasmid was linearized with *Eco*RI before transcription with T7 polymerase. To probe intron 2 and exon 3 of the ICP0 gene, a 760-bp *Xho*I-*Nru*I fragment from pRS-1 was cloned into the *Sal*I and *Sma*I sites of pGEM-1. The plasmid was digested with *Eco*NI before transcription with T7 polymerase, which resulted in a transcript of 336 nucleotides (nt) which spans a portion of intron 2 and exon 3. For the UL15 gene, a 270-bp *Xho*I-*Bst*EII

FIG. 1. RNase protection analysis of ICP0 mRNA in the presence of wild-type ICP27 or a repressor mutant. Total RNA was isolated from transfections with the plasmid pRS-1, which contains the genomic HSV-1 ICP0 gene, or with pDS-16, which contains a cDNA of the ICP0 gene (70). These target plasmids were cotransfected with a plasmid encoding the wild-type ICP27 gene (pSG130B/S) or the repressor mutant S18 (pS18) (23). RNase protection analysis was performed using a 32 P-labeled ICP0 antisense probe which spanned the junction of intron 1 and exon 2 and is shown schematically in Fig. 2A. Linearization of the T7 expression vector with *Eco*RI resulted in the transcription of an antisense RNA containing about 60 nt of vector sequence not found in the ICP0 gene so that undigested probe could be distinguished from fully protected precursor mRNA. The size of the spliced product was 203 nt and that of the precursor was 250 nt. To ensure that equal amounts of RNA were recovered and processed from each transfection, 1/10 of the RNA from each sample was hybridized with a probe specific for β -actin. Size markers (M) were obtained by end labeling *Hinf*I-digested pBR322 DNA. Appropriate exposures of the autoradiograms were scanned by a laser densitometer to quantify the amount of ICP0 mRNA in each lane.

fragment which spans the 3' splice site of UL15 was cloned into the *Sal*I and *Sma*I sites of pGEM1 after appropriate modification of the ends. This plasmid was linearized with *Hind*III before transcription with T7 polymerase.

Cell lines and viruses. HeLa spinner cultures and HeLa R19 monolayer cultures were grown in minimal essential medium supplemented with 5% newborn calf serum. Rabbit skin fibroblast cells, which were used for the transfections shown in Fig. 1, were grown as described previously (23). The HSV-1 wild-type strain KOS 1.1, the ICP27 temperature-sensitive mutant tsLG4, and the ICP27 mutant 27-LacZ, which has an insertion of the *lacZ* gene in the ICP27 locus, were described previously (57, 58).

Preparation of splicing extracts and *in vitro* splicing reactions. Spinner cultures (10^9) or monolayer cultures (7×10^7) of HeLa cells were either mock infected or infected with HSV-1 at a multiplicity of infection of 10 at the appropriate temperature. Nuclear extracts were prepared at various times after infection, as indicated in each experiment by the protocol described by Dignam et al. (9), or by using a small-scale extraction method (29). *In vitro* splicing reactions were performed as described by Krainer et al. (26) with the following modifications. Splicing reaction mixtures consisting of 50% (vol/vol) nuclear extract and 2×10^5 cpm of 32 P-labeled pre-mRNA were incubated at 30°C for 90 min. Splicing products were separated on denaturing 5% urea-polyacrylamide gels.

RNA isolation and RNase protection. Total RNA was extracted from infected or transfected cells by the guanidine thiocyanate method (4). Nuclear and cytoplasmic RNA fractions were isolated as described previously (54). RNA samples 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 or plasmid DNA had been removed from the samples before the RNase protection assays were performed. Antisense RNA was transcribed with T7 polymerase in the presence of [32 P]CTP. Equal amounts of the RNA samples (usually 25 μ g) were mixed with 5×10^5 cpm of antisense RNA, which had been gel purified. Hybridizations and RNase protections were performed essentially as described by Zinn et al. (71), except that the hybridization temperature used was 58°C because of the high GC content of the ICP0 and UL15 genes.

RESULTS

We previously showed that chloramphenicol acetyltransferase (CAT) mRNA levels were reduced 5- to 10-fold when ICP27 was present in transfections with CAT target plasmids containing either a 3' or 5' intron, regardless of the promoter sequences (54). Furthermore, if the introns were removed from the target genes, ICP27 had no effect on CAT mRNA. Before analyzing mRNA accumulation for viral genes which contain introns, we wanted to verify that ICP27 effects in fact depended on the presence of splicing signals. We transfected cells with a plasmid containing the viral ICP0 gene, which is 3,587 bp long and is composed of three exons (43). ICP0 is an immediate-early gene product, like ICP27, and it has been shown to act as a promiscuous transactivator in transfections (11, 12, 14, 20, 40, 41). We also used a plasmid which contains the complete cDNA of the ICP0 gene (pDS-16) and which includes identical regulatory sequences (70). The genomic ICP0 plasmid and the cDNA clone (pDS-16) were transfected into rabbit skin fibroblast cells along with a plasmid expressing the wild-type ICP27 gene or with a plasmid containing the ICP27 repressor mutant S18 (Fig. 1). The repressor region of ICP27, which encompasses the C-terminal region of the protein, is required for the effects which ICP27 has on the accumulation of spliced mRNAs, both in transfection and in infection (22, 54). RNase protections were performed using an ICP0 antisense RNA probe which spans the junction of intron 1 and exon 2 (shown schematically in Fig. 2). There was approximately 25 times as much ICP0-spliced mRNA (203-nt protected product) found in the transfection with mutant S18 as wild-type ICP27, whereas levels of ICP0 mRNA detected in S18 transfections were within twofold of the levels found with wild-type ICP27 when the DS-16 cDNA clone was used (Fig. 1, left-hand panel). Unspliced pre-mRNA (250-nt product) was detected in transfections with the genomic ICP0 clone and was found in a significantly greater ratio when wild-type ICP27 was expressed. This can be seen in the darker exposure of the autoradiograph shown in the right-hand panel of Fig. 1. In the transfection with wild-type ICP27, there was about 4 times as much ICP0 product as precursor found, whereas nearly 20 times as much product as precursor was seen with the repressor mutant S18. Normally, precursor RNA is difficult to detect because it is rapidly converted to spliced mRNA or is degraded. The ratio of product to pre-mRNA seen in the S18 transfection was nearer to what would be expected, that is, a much greater amount of spliced product relative to pre-mRNA was seen. In contrast, the ratio was significantly different in the presence of wild-type ICP27. Nearly the same amount of pre-mRNA was seen in the presence of wild-type ICP27 as that

FIG. 2. Accumulation of ICP0 pre-mRNA during HSV-1 infection. (A) Schematic representation of the ICP0 gene showing the regions of intron 1 and exon 2 which were spanned by the antisense RNA probe used in the protection analysis. The thickened line in the 310-nt probe represents the contribution from transcription vector sequences. (B) Total RNA was isolated from HeLa cells infected with wild-type HSV-1 KOS or the ICP27 null mutant, 27-LacZ, at the times indicated. RNase protection assays were performed using a 32 P-labeled ICP0 antisense probe shown in panel A, which yields a 250-nt product protected by pre-mRNA and a 203-nt spliced product. In addition, an RNA probe complementary to 28S rRNA was used as an internal standard to ascertain if equivalent amounts of total RNA were analyzed. The size of the rRNA protected product was 143 nt. UN, uninfected. (C) Nuclear (N) and cytoplasmic (C) fractionation was performed on HeLa cells infected with KOS for 6, 9, and 12 h. RNase protection analysis was performed with the antisense probe shown in panel A. DNA size markers consisted of end-labeled pBR322 digested with *Hinf*I. Appropriate exposures of the autoradiographs were scanned with a laser densitometer to quantify the amount of protected product in each lane.

with mutant S18, suggesting that transcription of the ICP0 genomic clone was equivalent with wild-type or mutant ICP27 present; however, greatly decreased levels of spliced product were found with wild-type ICP27. These results indicate that processing of the precursor to spliced product was defective when wild-type ICP27 was present.

Accumulation of unspliced pre-mRNA occurs during infection when ICP27 is expressed. To examine the effect that impairment of splicing would have on HSV-1 transcripts which are spliced, we measured the accumulation of product and precursor RNA for two viral genes, ICP0 and UL15. UL15 is a late gene product (5) which appears to play a role in DNA packaging (44). We previously found that ICP0 pre-mRNA could be detected at 4 h after infection and that expression of ICP27 during infection was required for the detection of pre-mRNA (22). To extend these observations throughout infection and to compare nuclear and cytoplasmic accumulations, we first isolated total RNA at different times throughout infection and then, in a second experiment, isolated nuclear and cytoplasmic RNA fractions. RNase protections were performed using antisense RNA probes which spanned the intron 1-exon 2 junction of each transcript (Fig. 2A and 3A). In the total RNA samples, it was found that ICP0 pre-mRNA could be detected in significant amounts in the wild-type KOS-infected cells from 6 h after infection, increasing up to 12 h after infection (Fig. 2B). ICP27 protein levels which are low at early times after infection also increase as infection proceeds. Significantly, ICP0 pre-mRNA was undetectable or barely detectable in infections with the ICP27 mutant 27-LacZ (Fig. 2B). Pre-mRNA was also detected for UL15, and, in fact, higher amounts of precursor than product RNA were found in the total RNA samples (Fig. 3B). UL15 is a late gene product, and ICP27 is required for abundant HSV-1 late gene expression; therefore, the comparable infection with the 27-LacZ null mutant was not performed. These data show that when ICP27 was expressed during infection, precursor RNA was found to accumulate for two viral spliced products and that the accumulation increased as the infection proceeded, during which time ICP27 levels were increasing. Greater accumulation of pre-mRNA was seen during infection (Fig. 2 and 3) than during transfection (Fig. 1). This could indicate that infection compromises the ability of the nucleus to rapidly degrade unprocessed transcripts, perhaps as a result of high rates of transcription of unspliced HSV-1 mRNAs.

To analyze nuclear and cytoplasmic accumulations of product and precursor mRNA, infected cells were fractionated at different times after infection with wild-type KOS. While pre-mRNA could be detected in the cytoplasm for both ICP0 (Fig. 2C) and UL15 (Fig. 3C), especially at 12 h after infection, the accumulation of pre-mRNA in the nucleus was substantially greater for both transcripts. For example, densitometry scanning indicated that the product-to-precursor ratio for ICP0 nuclear RNA was about 1:1 at 9 and 12 h after infection, whereas there was about six times as much product as pre-mRNA detected in the cytoplasm at 9 h after infection (Fig. 2C). Similarly, about twice as much UL15 pre-mRNA as product was found in the nucleus at 12 h after infection, with the reverse ratio occurring in the cytoplasm (Fig. 3C). In fact, the amount of pre-mRNA recovered in the cytoplasmic fractions may be an overestimate of the actual amount present. This is because viral packaging is occurring by 9 to 12 h after infection and, therefore, significant amounts of viral structural proteins are being transported to the nucleus, making fractionation of the cytoplasm that is free from nuclear contamination difficult. Nevertheless, these data show that during wild-type HSV-1 infection, pre-mRNA for two viral transcripts accumu-

FIG. 3. Accumulation and distribution of UL15 pre-mRNA during infection. (A) The antisense RNA probe used is shown schematically, along with the HSV-1 UL15 gene. The UL15 precursor RNA product spanning the junction of intron 1 and exon 2 was expected to be 270 nt, while the spliced product was 205 nt. (B) Total RNA isolated from HeLa cells infected with HSV-1 KOS for 3, 6, 9, and 12 h was analyzed by RNase protection. UN, uninfected. (C) Nuclear (N) and cytoplasmic (C) fractions were prepared from HeLa cells infected with KOS at the times indicated. The RNA was analyzed by RNase protection using the antisense RNA probe shown in panel A.

lated in the nucleus, which suggests that splicing is affected and that ICP27 does not facilitate transport of pre-mRNA.

The presence of two introns in the ICP0 gene suggests that alternatively spliced products could arise. Splicing of the first

FIG. 4. Accumulation and distribution of unspliced ICP0 pre-mRNA in wild-type- and 27-LacZ-infected cells. Nuclear (N) and cytoplasmic (C) fractions were prepared from cells infected with KOS or 27-LacZ at the times indicated. The RNA was analyzed using a probe which was antisense to a portion of ICP0 intron 2 and exon 3 as described in Materials and Methods. The fully protected pre-mRNA product was expected to be 336 nt, and the fully spliced product was 200 nt. An antisense 28S rRNA probe was included in the protection assays to determine if equivalent amounts of RNA were analyzed. The size of the rRNA protected product was 143 nt.

but not the second intron would result in an ICP0 product of 262 amino acids because an in-frame stop codon occurs within intron 2. Transfection studies using a plasmid constructed to express this product showed that the 262-amino-acid protein had a dominant-negative effect on gene expression and virus infection (68, 69). Everett et al. (13) showed that the predicted truncated product of ICP0 is expressed at low levels during normal HSV-1 infection. Because ICP0 pre-mRNA was found to accumulate during wild-type infection, it was of interest to determine if higher amounts of ICP0 pre-mRNA retaining intron 2 were found in the cytoplasm in which translation would yield the truncation product. In this experiment, infected cells were fractionated into nuclear and cytoplasmic fractions at earlier times after infection compared with the experiment in Fig. 2 to avoid possible nuclear contamination of the cytoplasmic fractions. RNase protections were performed with a probe which spans a portion of intron 2 and exon 3. As was seen previously in Fig. 2C, significant amounts of pre-mRNA were found to accumulate in the nucleus by 7 h after infection (Fig. 4). At this time, the precursor-to-product ratio in KOS-infected cells was about 1 to 1 in the nuclear fraction. Very little pre-mRNA was found in the cytoplasmic fractions, again demonstrating that splicing rather than transport appears to be affected in wild-type HSV-1 infection. In accord with results in Fig. 2B, little or no pre-mRNA could be detected in infections with 27-LacZ, either in nuclear or cytoplasmic fractions, indicating that processing was not impaired when ICP27 was not expressed.

Although pre-mRNA was seen to accumulate in the nucleus, some pre-mRNA was detected in the cytoplasmic fractions. In addition, incompletely spliced mRNA appears to be transported to the cytoplasm, as indicated by the finding by Everett et al. (13) that the truncation product comprised up to 5% of ICP0 protein. To confirm that precursor RNA entered the cytoplasm and that this pre-mRNA did not result from nuclear contamination, an experiment was performed to assess the stability of this RNA. Pre-mRNA that is not processed is

FIG. 5. Stability of ICP0 pre-mRNA. Infections were performed with wild-type KOS for 5 h, at which time nuclear (N) and cytoplasmic (C) fractionation was performed on one set of infected cultures (lanes 1 and 2), and actinomycin D (Act D; 10 μ g/ml) was added to parallel sets of cultures (lanes 3 to 6). Fractionation and RNA extraction were performed 1 h (lanes 3 and 4) and 2 h (lanes 5 and 6) after the addition of actinomycin D. RNase protection assays were performed using an ICP0 probe which spanned the junction of intron 2 and exon 3 as described in the legend to Fig. 4.

rapidly degraded in the nucleus. On the other hand, unspliced RNA that does enter the cytoplasm might have stabilities similar to those of spliced mRNA (6, 32, 42), provided that cytoplasmic destabilization elements (8) are not present in the intron sequences. To determine the stability of ICP0 pre-mRNA in the nucleus and cytoplasm, infections were performed with wild-type KOS for 5 h, at which time cells were fractionated and RNA was extracted from one set of cultures, and actinomycin D was added to parallel sets of infected cultures. RNA was extracted 1 and 2 h after the addition of actinomycin D. Figure 5 shows the results of the RNase protection analysis on nuclear and cytoplasmic RNA fractions using the ICP0 probe that spans the junction of intron 2 and exon 3. Pre-mRNA was detected at 5 h in both nuclear and cytoplasmic fractions, although the relative ratio was higher in the nuclear fraction, as expected. One hour after treatment with actinomycin D, pre-mRNA was still found in the cytoplasmic fraction, whereas it was barely detectable in the nuclear fraction. Pre-mRNA in the nucleus was either processed or degraded during the actinomycin D block. Therefore, the pre-mRNA seen in the cytoplasmic fraction appeared to represent unspliced RNA that entered the cytoplasm rather than nuclear contamination of the cytoplasmic fraction.

Impairment of splicing occurs early during HSV-1 infection and appears to require ICP27 expression. The data presented thus far suggest that processing of spliced mRNA is impaired during infection with wild-type HSV-1. To determine directly whether HSV-1 infection inhibits host cell splicing and to ascertain whether ICP27 is required for the inhibition, we performed *in vitro* splicing reactions in nuclear extracts prepared from infected and uninfected cells. HeLa cells were either mock infected or infected with HSV-1 wild-type KOS or

FIG. 6. Analysis of the splicing activities of nuclear extracts isolated from uninfected (UN) or HSV-1-infected cells. Nuclear extracts were prepared from uninfected or KOS- or 27-LacZ-infected HeLa cells at 3.5 and 6 h after infection. In vitro splicing reactions were performed at 30°C for 90 min using a 32 P-labeled β -globin pre-mRNA substrate derived by SP6 transcription of plasmid pSP64-H β 86. Control reactions lacking nuclear extract (-NE) were also performed. The positions of the unspliced substrate RNA (497 nt) the spliced product (370 nt) and splicing intermediates (intron/lariat-exon 2, 380 nt; exon 1, 155 nt; and intron/lariat, 143 nt) are shown schematically on the right. DNA size markers (M) consisted of 32 P-labeled *Hinf*I-digested pBR322 DNA.

the ICP27 mutant 27-LacZ. Cells were harvested and nuclear extracts were prepared at 3.5 and 6 h after infection. A 32 P-labeled β -globin RNA splicing substrate transcribed from the plasmid pSP64H β 86 (26) was used in the in vitro splicing reactions. As measured by densitometry, a 30 to 50% conversion of pre-mRNA to spliced product was found for extracts from uninfected cells and from 27-LacZ-infected cells (Fig. 6, lanes 1, 5, and 6). In contrast, extracts from wild-type KOS-infected cells showed little evidence of splicing (Fig. 6, lanes 2 and 3). Furthermore, the absence of spliced product or intermediates in the extracts from KOS-infected cells did not appear to be due to the degradation of RNA, since the substrate pre-mRNA was stable during the course of the experiment. Although this result has been repeated several times, some variability in the degree of inhibition of splicing in extracts from KOS-infected cells has occurred. In some extracts, a 10 to 15% conversion of pre-mRNA to product was seen, while in most extracts from KOS-infected cells, spliced products were barely detectable. The extracts which showed some conversion were made from cells infected with KOS for times longer than 6 h, that is, 9 to 12 h after infection. It is possible that ICP27 is modified differentially at later times in infection, thus altering its effect on splicing. However, we have

FIG. 7. Effect of mixing competent splicing extracts with a splicing-defective extract from KOS-infected cells. Nuclear extracts were prepared from uninfected (UN) or KOS- or 27-LacZ-infected HeLa cells. Splicing reactions were performed to determine the splicing activity of each extract (lanes 2 to 4). Reactions were performed as described in the legend to Fig. 4. To determine if the inhibitory activity seen in the extract from KOS-infected cells could decrease splicing in extracts from uninfected and 27-LacZ-infected cells, 7.5 μ l of the extract from KOS-infected cells was mixed with an equal amount of extract from uninfected cells and from 27-LacZ-infected cells. Either splicing reactions were performed immediately after mixing of the extracts (lanes 5 and 8), or the mixed extracts were preincubated at 30°C for 60 min (lane 6) or 120 min (lanes 7 and 9), at which time the β -globin RNA substrate was added and the splicing reactions were performed. To determine whether preincubation alone affected the splicing activities of the extracts, uninfected and KOS- and 27-LacZ-infected cell extracts were similarly incubated at 30°C for 120 min before splicing reactions were performed (lanes 10 to 12). Control reactions lacking nuclear extract (-NE [lane 1]) were also performed.

consistently found that extracts from 27-LacZ-infected cells are active in splicing in a manner equivalent to that of extracts obtained from uninfected cells. Thus, infection with wild-type HSV-1 appears to inhibit host cell splicing, and ICP27 expression is required for this effect to occur.

The inhibition in splicing found in extracts from wild-type HSV-1-infected cells can be imparted to extracts competent in splicing. To determine whether the inhibitory activity in wild-type-infected cells could be transferred to competent splicing extracts, biochemical complementation assays were performed. Extracts were prepared from uninfected or HSV-1 KOS- or 27-LacZ-infected HeLa cells. First, the three extracts were tested for splicing ability. As shown in Fig. 7, lanes 2 to 4, when splicing reactions were performed without preincubation of the extracts (0'), results similar to those described above were found. That is, uninfected and 27-LacZ-infected extracts were efficient at splicing the substrate, whereas KOS extracts showed no detectable splicing. Extracts from KOS-infected cells were then mixed in a 1:1 ratio with extracts from 27-LacZ-infected cells or uninfected cells. The mixed extracts were either used immediately for splicing reactions (0' [Fig. 7, lanes 4 and 8]), or were preincubated at 30°C for 60 (lane 6) or 120 (lanes 7 and 9) min and then used for splicing reactions. In mixed extracts, the efficiency of splicing was reduced immedi-

ately upon mixing, but the reduction in splicing became more pronounced with time of incubation of the mixed extracts before the splicing reactions (lanes 6, 7, and 9). Importantly, the splicing efficiency of uninfected or 27-LacZ-infected extracts which were not mixed with KOS extracts did not change with a 2-h incubation before the splicing reactions (lanes 10 and 12). These results suggest that some activity in extracts from KOS-infected cells can interfere with splicing in extracts which are otherwise competent in splicing. Further, this activity requires time to fully accomplish the inhibition.

Restoration of ICP27 activity inhibits splicing in a competent extract. Next, we sought to determine whether the inhibition of splicing seen in extracts from KOS-infected cells was due to the activity of the ICP27 protein directly or indirectly because of the action of some other viral product which requires ICP27 for its expression. To address this question, an ICP27 temperature-sensitive mutant, *ts*LG4, was used to infect cells, because we previously showed that ICP27 protein made during *ts*LG4 infection at nonpermissive temperature could regain activity and function in vivo when cultures were shifted to 33°C (22a). Nuclear extracts were prepared from HeLa cells infected with *ts*LG4 at 33°C (permissive temperature) or 39°C (nonpermissive temperature). Either these extracts were used immediately in splicing reactions (Fig. 8, lanes 2 and 4), or the extracts were preincubated for 2 h at 30°C before addition of the splicing substrate. The preincubation was performed to allow renaturation of ICP27 made during infection at 39°C. As expected, extracts from cells infected with *ts*LG4 at 33°C, at which ICP27 is functional, showed little evidence of splicing with or without preincubation of the extract (lanes 2 to 3). In contrast, splicing of the substrate pre-mRNA was seen in the extract from cells infected with *ts*LG4 at 39°C, at which ICP27 is not functional (lane 4). However, when a portion of this same extract was preincubated at 30°C for 2 h before addition of the β -globin mRNA substrate, splicing activity was greatly diminished (lane 5) compared with the activity seen without preincubation (lane 4). Longer incubation (3 h) at 30°C did not result in complete inhibition (data not shown), suggesting that even after renaturation, the mutant protein cannot regain full wild-type function. No reduction in splicing activity was seen in uninfected extracts treated identically (lanes 1 and 6). Mixing a portion of the *ts*LG4 39°C extract with uninfected extract and then preincubating at 30°C for 2 h before performing the splicing reaction also resulted in a decrease in spliced products (data not shown), demonstrating that the renatured inhibitory activity could be imparted to a splicing-competent extract. These results show that the inhibitory effect on splicing seen when ICP27 was functional was due to the activity of ICP27. This is because during preincubation of the extract no protein synthesis occurred, and viral products requiring ICP27 for expression could thus not be made. Furthermore, when ICP27 regained activity, splicing was impaired. However, these data do not exclude the possibility that ICP27 could alter another viral protein posttranslationally, which could then affect splicing.

DISCUSSION

HSV is an unusual eukaryotic DNA virus in that the majority of its more than 70 transcripts are not spliced (65). In fact, only five HSV-1 genes expressed during lytic infection and one gene expressed during latency have been shown to be spliced (5, 19, 43, 66, 67). Three of the lytically expressed genes (ICP0, ICP22, and ICP47) are immediate-early genes, and two (UL15 and glycoprotein C) are late genes. Interestingly, four of these gene products (ICP0, ICP22, ICP47, and gC) are not strictly

FIG. 8. Reactivation of ICP27 activity in extracts from *ts*LG4-infected cells. HeLa cells were infected with the ICP27 temperature-sensitive mutant *ts*LG4 at 33°C (permissive temperature) or at 39°C (nonpermissive temperature). Nuclear extracts were prepared 3 h after infection. The extracts were either used immediately for splicing reactions (lanes 2 and 4) or were preincubated for 2 h at 30°C, at which time the RNA splicing substrate was added and the splicing reactions were performed (lanes 3 and 5). Control reactions were also performed with extracts from HeLa cells which were mock infected and incubated at 39°C for 3 h, at which time the nuclear extract was prepared. As with the extracts from *ts*LG4-infected cells, either splicing reactions were performed immediately (lane 1) or the extract was preincubated at 30°C for 2 h before the splicing reaction was performed (lane 6). UN, uninfected.

required for viral replication in tissue culture (25, 34, 45, 53, 59). In addition, evidence has been presented previously that HSV-1 infection may actually inhibit host cell splicing. In these studies, an accumulation of unspliced host precursor RNA was found (55), or decreased accumulation of host spliced mRNAs was seen. Furthermore, a viral immediate-early protein, ICP27, has been shown to be required for some of these effects to occur (22). To determine what effect this proposed inhibition of splicing would have on viral products which were spliced, we monitored the nuclear and cytoplasmic accumulations of pre-mRNA and spliced products for two viral transcripts, an immediate-early product (ICP0) and a late product (UL15). Further, we measured in vitro splicing activities of nuclear extracts from HSV-1-infected cells compared with uninfected cell extracts to directly determine whether splicing is inhibited following HSV-1 infection.

Unspliced pre-mRNA was detected in KOS-infected cells for both ICP0 and UL15 (Fig. 2 and 3). Pre-mRNA was not detected or was barely detectable in infections with a viral mutant which does not express ICP27 (Fig. 2 and 4). The amount of pre-mRNA detected in the nuclear fraction of

wild-type-infected cells was considerably greater for both transcripts and increased with time after infection. These data indicate that splicing and not RNA transport was affected. This result is different from that found with the human immunodeficiency virus Rev protein, which facilitates transport of incompletely spliced or unspliced viral mRNAs encoding late gene products (6). Malim et al. (32) measured the ratio of unspliced to spliced *env* RNA in nuclei and cytoplasm. They found that Rev had little effect on the ratio of unspliced to spliced *env* RNA in the nucleus. In contrast, we found a greater ratio of unspliced to spliced RNA in the nucleus than in the cytoplasm, and unspliced RNA did not accumulate in the absence of ICP27. Despite the accumulation of precursor RNA, abundant levels of spliced ICP0 transcripts were found. This result is also different from that found for the influenza virus NS1 protein which affects nuclear export of mRNA and also inhibits splicing (1, 18, 31, 46, 47). NS1 protein inhibits the transport of mRNAs (1, 18) by binding to the poly(A) sequence at the 3' ends of mRNAs, resulting in the inhibition of nuclear export of all poly(A)-containing viral and cellular mRNAs (47). ICP27 does not appear to facilitate the transport of unspliced RNA, nor is transport of poly(A)-containing mRNA blocked. Interestingly, Weber and Wigdahl (69) have shown that a mutant version of ICP0 which contains the first two exons but not the third can act as a dominant negative mutant and interfere with expression from a variety of HSV-1 promoters in transfection assays. A similar mutant protein would be formed by translation of an incompletely spliced ICP0 mRNA which retained the second intron, and Everett et al. (13) detected this truncation product in wild-type-infected cells. We were able to detect small amounts of unspliced ICP0 RNA in the cytoplasm of infected cells using a probe which spanned the junction of intron 2 and exon 3 (Fig. 4 and 5). However, the amounts of pre-mRNA detected with probes which spanned intron 1 or intron 2 were similar, which suggests that ICP27 expression does not affect alternative splicing but appears to decrease splicing efficiency in general.

The two late transcripts which are spliced, UL15 and gC, are spliced poorly giving rise to large amounts of unspliced RNA (Fig. 3) (19). In human immunodeficiency virus, late products are translated from incompletely spliced or unspliced mRNAs, and thus it is important that these RNAs be transported to the cytoplasm. In HSV-1, the spliced UL15 product is translated to yield functional protein (2). Although splicing of this product is inefficient, sufficient levels are obviously produced to allow successful packaging of viral DNA (44). In the case of gC, splicing is undesirable because the unspliced mRNA encodes the coding sequence (19). It was not possible to compare splicing of late transcripts in the absence of ICP27 as was done for ICP0 because functional ICP27 is required for HSV-1 late gene expression. This requirement is independent of any effects of ICP27 on splicing but instead depends on its other effects on transcription (35), 3' processing (36), and DNA replication (7). In summary, impairment of splicing appears to have few benefits or consequences for the expression of HSV-1 genes which are spliced.

In the second part of this study, we measured the effect of HSV-1 infection on splicing *in vitro* using nuclear extracts from uninfected or infected cells. Splicing of a β -globin substrate RNA occurred at an efficiency of about 30 to 50% conversion from precursor to fully spliced product in extracts from uninfected HeLa cells or from cells infected with the ICP27 null mutant 27-LacZ. In contrast, spliced products were barely detectable in extracts from cells infected with wild-type HSV-1 KOS. These data indicate that HSV-1 infection does inhibit host cell splicing and corroborate results which showed nuclear

accumulation of cellular precursor mRNA (55) or decreased accumulation of spliced cellular mRNAs (22). In addition, these data also show that ICP27 expression is required for the inhibition of host cell splicing. It was also shown that ICP27 itself was required for the inhibition, rather than some other viral product which requires ICP27 for its expression. That was shown by allowing a conformationally defective form of ICP27 synthesized in *ts*LG4-infected cells to renature *in vitro*. While this extract was competent for splicing when the reactions were performed without additional treatment, preincubation of the extract at 30°C resulted in an abolition of splicing activity. This result confirms that ICP27 itself is responsible for the inhibitory activity. Additional support for this conclusion comes from preliminary results in our laboratory that showed that purified baculovirus-expressed ICP27 inhibited splicing when it was added to uninfected cell extracts (23a). This is also in accord with our previous study which showed that ICP27 was required for the decreased accumulation of host cell spliced mRNAs after infection (22). Furthermore, in that study we found that the C-terminal repressor region of the protein must remain intact for the reduction in spliced mRNAs to occur. The data presented here also support that finding. Namely, in transfections with an ICP27 repressor mutant, S18, decreased accumulation of spliced ICP0 mRNA was not seen (Fig. 1). In addition, the lesion in *ts*LG4 maps to the repressor region of the protein (58).

Differences in the extents of inhibition of splicing were noted between *in vivo* levels of spliced products detected during transfection or infection and *in vitro* levels found in extracts from wild-type-infected cells. Thus, in the presence of ICP27, spliced mRNAs were decreased 5- to 20-fold in transfections (Fig. 1) (54) and during infection (22). However, we have consistently observed greater reductions in splicing using extracts from KOS-infected cells compared with extracts from uninfected or 27-LacZ-infected cells. This may be due to the fact that during the *in vitro* reaction, a single substrate is presented to the splicing machinery, so that inhibitory effects would be exacerbated compared with cell cultures, in which thousands of transcripts are spliced at any given time.

While these experiments do not address the mechanism by which ICP27 inhibits splicing, the data presented in the biochemical complementation experiment shown in Fig. 7 suggest that the inhibitory activity of ICP27 acts on the protein level rather than by binding to substrate RNA. Performance of splicing reactions immediately after mixing extracts resulted in reduced splicing; however, greater inhibition was seen upon preincubation of the extracts for 1 to 2 h before addition of the RNA substrate. This would suggest that the inhibitory activity continued to function in the extracts. This was also suggested by the renaturation of the conformationally defective ICP27, which, upon incubation at 30°C, caused a decrease in the splicing activity of the extract. Another possible mechanism of inhibition involves binding to small nuclear RNA. It has been shown recently that influenza virus NS1 protein is associated with U6 small nuclear RNA in splicing extracts (31). The pre-mRNA forms spliceosomes; however, subsequent catalytic steps in splicing are inhibited because the NS1 protein remains associated with the spliceosomes. The RNA-binding domain of the NS1 protein is required for the inhibition of splicing by interacting with a sequence in U6 small nuclear RNA (31). ICP27 lacks a ribonucleoprotein-binding domain of the RNA recognition motif type (33), and, while it contains an arginine-rich region which functions in nuclear localization (53a), it is not an arginine-serine domain. We have shown that ICP27 binds single-stranded DNA nonspecifically (64); however, we have not yet shown RNA binding, and we have not demon-

strated which region of the protein is involved in the single-stranded DNA binding. The region of ICP27 which appears to be essential to its inhibitory effect on splicing is what we have termed the repressor region of the molecule. This C-terminal region contains a single zinc finger-like motif, and we have shown that ICP27 binds zinc in vitro and that the repressor region of the protein is required for this binding (64). A number of spliceosome proteins have been shown to contain a single zinc finger motif. These include the mammalian factor SAP 62 (3), as well as the yeast proteins PRP9 and PRP11 (30). In addition, preliminary experiments analyzing spliceosome formation in extracts from KOS-infected cells have shown that, unlike the situation with NS1 protein, higher-order, ATP-dependent complex formation was diminished (23a).

Future studies will be directed toward biochemical fractionation and elucidation of the stage(s) in splicing or spliceosome assembly which is affected by the action of ICP27. These studies are of general importance because they provide an additional probe to dissect the complex process of mammalian splicing and because ICP27 may function by a novel mechanism in inhibiting RNA splicing.

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