A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing

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Post-transcriptional control mechanisms play an important role in regulating gene expression for a number of viruses, especially in the regulation of late gene products. In this study we have investigated the mode of action of ICP27, an immediate-early regulatory protein of herpes simplex virus 1 (HSV-1) required for late gene expression. Transfection experiments have demonstrated that ICP27 can activate or repress expression depending on the target gene. Here, we show that the regulatory activity of ICP27 is independent of the target gene promoter sequences but, instead, depends on the presence of different mRNA processing signals. The activation function correlated with different polyadenylation sites, whereas the repressor function correlated with the presence of introns either 5' or 3' to the target gene-coding sequences. Poly(A)⁺ RNA levels were increased by ICP27 in transfections with a target gene having only an AATAAA recognition signal but no G/U box within the usual distance. In contrast, 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. These results suggest that this essential HSV-1 regulatory protein acts post-transcriptionally to affect mRNA processing and point to possible interactions between splicing and polyadenylation factors.

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The regulation of gene expression in eukaryotic cells is controlled at several levels, including post-transcriptionally. The steady-state level of a specific mRNA can be regulated during processing, including polyadenylation and splicing (Leff et al. 1986), during transport from the nucleus to the cytoplasm (Cullen and Greene 1989), and by degradation of mature RNA in the cytoplasm (Atwater et al. 1990). A number of DNA viruses and retroviruses use post-transcriptional mechanisms, particularly in the regulation of late gene expression during lytic growth. The adenovirus late transcription unit dominates at late times during infection producing up to 10% of total poly(A)⁺ mRNA, which encodes the majority of the viral structural proteins (Lucas and Ginsberg 1971; Shaw and Ziff 1980). Transcripts that arise from the major late promoter can be processed at any one of five possible poly(A) sites (Shaw and Ziff 1980; Nevins and Wilson 1981), as well as multiple splice acceptor sites (Berget et al. 1991; Nevins and Wilson 1981; Adami and Nevins 1988; Chow et al. 1991). Polyomavirus late nuclear transcripts are larger than genome in length and heterogeneous as a result of a decreased efficiency of termination that occurs at late times (Hyde-DeRuyscher

and Carmichael 1988). Processing into mature late viral mRNAs involves the splicing of noncoding leader exons to one another and to a coding exon (Barrett et al. 1991). In cytomegalovirus, a human herpesvirus, post-transcriptional events affect the regulation of all classes of viral genes (DeMarchi 1983; Goins and Stinski 1986; Geballe and Mocarski 1988; Stamminger et al. 1991); however, several late transcripts have been shown to differ from early transcripts by processing of the 3' ends involving either alternate poly(A) site cleavage or differential splicing of the late transcripts (Goins and Stinski 1986). Human immunodeficiency virus (HIV-1) also uses post-transcriptional controls for late gene expression. In this case, the viral product *rev* acts to induce the cytoplasmic accumulation of incompletely spliced HIV-1 mRNAs that encode the viral structural proteins by promoting the nucleocytoplasmic transport of these RNAs (Chang and Sharp 1989; Emerman et al. 1989; Felber et al. 1989; Malim et al. 1989). Adenovirus also encodes a polypeptide of the E1B gene that, by facilitating an intranuclear step in the metabolism of viral late mRNAs, results in their improved transport to the cytoplasm (Leppard and Shenk 1989). Another post-transcriptional mechanism, variation in mRNA stability, is used by human papillomavirus type 16 (HPV16) in regulating late

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gene expression (Kennedy et al. 1991). A negative regulatory element in the HPV16 genome located immediately upstream of the mRNA 3'-processing signals acts as a late mRNA instability element (Kennedy et al. 1991).

We have been studying the mode of action of the human herpes simplex virus type 1 (HSV-1) immediate-early protein ICP27 (IE63) and its role in the switch from early to late expression during infection. ICP27 is a 63-kD protein that localizes to the nucleus (Ackerman et al. 1984; Knipe and Smith 1986; Hardwicke et al. 1989), is phosphorylated (Wilcox et al. 1980), and is essential for viral replication (Sacks et al. 1985). Studies with ICP27 temperature-sensitive and null mutants have demonstrated that this protein is involved in the negative regulation of viral immediate-early and early genes, and host cellular genes, and is required for the expression of HSV-1 late genes (Sacks et al. 1985; McCarthy et al. 1989; McMahan and Schaffer 1990; Rice and Knipe 1990). To assess directly the regulatory activities of ICP27 on HSV-1 target genes, we performed transfection experiments with the cloned ICP27 gene as an effector plasmid, and target plasmids consisting of HSV-1 promoter sequences fused to the chloramphenicol acetyltransferase (CAT) gene (Sekulovich et al. 1988). These experiments showed that ICP27 could repress or activate expression depending on the target plasmid in accord with its role during infection as a negative regulator of early expression and as an activator of late gene expression. Surprisingly, during these transfection studies, we found that ICP27 functions as a regulator in a promoter-independent manner. Specifically, target plasmids with identical promoter sequences but different reporter genes (CAT and *lacZ*) did not respond similarly to ICP27. This result prompted us to evaluate the differences in these reporter genes; and as described here, we found that dissimilar eukaryotic mRNA processing signals added to these reporter genes were responsible for the different effects seen with ICP27.

In this study we analyzed the ability of ICP27 to activate or repress expression of target genes having the same promoter sequences but with different polyadenylation regions or with the insertion of introns either 5' or 3' to the reporter gene-coding sequences. Activation of target gene expression by ICP27 was found with some of the polyadenylation signals used, whereas no change in the level of expression was seen with others. In contrast, repression of expression was seen with ICP27 when an intron was present in the target gene regardless of which poly(A) region was used. A mutant containing an insertion within the activation region of ICP27 (Hardwicke et al. 1989) was shown not to activate expression of those targets with specific poly(A) regions whose expression was increased by wild-type ICP27. Similarly, a mutant within the repressor region of ICP27 (Hardwicke et al. 1989) failed to repress expression of targets with introns. Analysis of nuclear and cytoplasmic RNA showed that the level of spliced CAT mRNA was reduced in transfections with ICP27, but the distribution of spliced products was similar, which suggests that ICP27 is not in-

involved in transport. Together, these results point to a post-transcriptional role for ICP27 at the level of mRNA processing.

Results

To assess the regulatory activities of ICP27, transfection experiments have been performed. In these studies, ICP27 was shown to have little or no effect on its own on target gene expression when HSV-1 early or late promoters were used in the target constructs. However, when ICP27 was added in combination with two other HSV-1 *trans*-activators, namely ICP4 and ICP0, either a repression of the activation seen with ICP4 and ICP0 or an augmentation of that activation was seen depending on the target gene (Everett 1986; Block and Jordan 1988; Rice and Knipe 1988; Sekulovich et al. 1988; Hardwicke et al. 1989; Rice et al. 1989; McMahan and Schaffer 1990). Thus, ICP27 repressed ICP4- and ICP0-activated expression of pTK-CAT, which contains the early promoter from the thymidine kinase gene, whereas it enhanced the expression of pVP5-CAT, which contains the late promoter from the major virion capsid gene (Sekulovich et al. 1988; Hardwicke et al. 1989). For this reason, transfection experiments with ICP27 are performed routinely in the presence of ICP4 and/or ICP0. By constructing a set of in-frame insertion and deletion mutations (Hardwicke et al. 1989), we showed further that the activation region of ICP27 maps to the carboxy-terminal half of the protein, that activation mutations are *trans*-dominant to the wild-type protein (Smith et al. 1991), and that the repressor region maps to the carboxy-terminal 70–80 amino acids of ICP27 (Hardwicke et al. 1989). During the course of these studies, we constructed target plasmids containing HSV-1 promoters fused to a second reporter gene, namely the *lacZ* gene encoding β -galactosidase, for the purpose of performing cotransfection experiments to determine whether ICP27 could activate expression of one target gene while repressing a second target within the same cell. In testing the *lacZ* targets, an unexpected result was found. As shown in Table 1 (columns 1 and 3), the ICP4- and ICP0-activated expression of pTK-CAT was repressed ~12-fold by adding ICP27 to the transfections, whereas ICP27 had no effect on the activated expression of pTK-LacZ. This result suggested that ICP27 was behaving as a reporter gene-dependent regulator because the TK promoter sequences were identical in both plasmids.

To analyze this promoter-independent effect of ICP27, we looked first at differences between the pTK-CAT and pTK-LacZ targets, besides protein-coding sequences. One striking difference was in the eukaryotic mRNA processing signals for expression in mammalian cells that had been added to the prokaryotic reporter genes. Thus, pTK-CAT (Sekulovich et al. 1988) derived from pSV2-CAT (Gorman et al. 1982) contained the small t-antigen intron and early poly(A) region from SV40, whereas pTK-LacZ, derived from pON1 (Spaete and Mocarski 1985) contained only the SV40 early poly(A) signal but no intervening sequences. Furthermore, we discov-

Table 1. Effects of ICP27 on the expression of different reporter genes containing the same promoter

Effectors	Fold induction ^a					
	pTK-CAT	pTK-CAT-SYN A	pTK-LacZ	pTK-CAT/pTK-LacZ	pTK-CT-SYN A/pTK-LacZ	
UN	1	1	1	1	1	1
ICP4 + 0	24.2	1.7	8.1	33.5	1.5	6.9
ICP4 + 0 + 27	2.1	7.5	7.9	3.6	17.7	11

The target plasmids pTK-CAT and pTK-CAT-SYN A, which contain the CAT reporter gene, and pTK-LacZ, which contains the *lacZ* reporter gene, were transfected into rabbit skin fibroblast (RSF) cells either singly (20 µg/ml) or in combination with each other (10 µg/ml for each target plasmid). The uninduced (UN) level of CAT activity or β-galactosidase activity found for each target plasmid in the absence of HSV-1 effectors was arbitrarily set at 1.0. Cotransfections with plasmids encoding the HSV-1 effectors ICP4 (pSG28K/B), ICP0 (pRS-1) and ICP27 (pSG130B/S) added in equimolar amounts were performed as described previously (Sekulovich et al. 1988).

^aThe level of CAT activity or β-galactosidase activity obtained in the presence of effector plasmids was calculated relative to the uninduced level.

ered that the CAT cassettes in the pTK-CAT and pVP5-CAT plasmids, which we had used as our model HSV-1 early and late target genes, were not the same. As described above, pTK-CAT was derived from pSV2-CAT; however, pVP5-CAT was derived from pSV0d(ori⁻)-CAT (Costa et al. 1985), a derivative of pUC-CAT described originally by Linney and Donerly (1983). These investigators inserted a synthetic oligonucleotide linker 3' to the CAT-coding sequences. This oligonucleotide contained the highly conserved polyadenylation recognition signal AATAAA (Proudfoot and Brownlee 1974); however, a second signal, termed the G/U box (Gil and Proudfoot 1984; McDevitt et al. 1984; McLauchlan et al. 1985), which is also required for efficient polyadenylation, was not included. Therefore, it was necessary to determine whether ICP27 activated pVP5-CAT in response to the late promoter or because of different mRNA processing signals compared with pTK-CAT. Accordingly, the TK promoter and the VP5 promoter were fused to the CAT gene containing the SV40 small t intron and early poly(A) signals (termed CAT) and to the CAT gene containing only the poly(A) hexanucleotide AATAAA (termed CAT-SYN A). In addition, the promoter from the glycoprotein B (gB) gene (Bzik et al. 1984), another HSV-1 late gene, and the SV40 early promoter and enhancer (Gorman et al. 1982) were fused to each CAT gene so that it could be determined whether the promoter played any role in the ICP27 response.

Transfections with each target plasmid were performed, and CAT activity was measured. In each case, the uninduced level obtained from transfections with the target plasmid alone in the absence of effector plasmids was set at 1.0, and fold induction was calculated relative to this value. For all of the promoters used, opposite effects were obtained with ICP27, depending on the CAT construct (Fig. 1). For example, an almost 10-fold reduction in the ICP4- and ICP0-activated response of pTK-CAT was seen with ICP27, whereas a 3-fold stimulation of that activation was seen for pTK-CAT-SYN A (Fig. 1). Similar results were seen for the CAT constructs with the gB, VP5, and SV40 promoters. When the CAT plasmid used contained the SV40 small t intron and early poly(A) signals, repression of ICP4- and ICP0-activated expression was seen. On the other hand, aug-

mentation of that activation was seen with the CAT-SYN A plasmids that lack the G/U box. It was also noted that ICP4- and ICP0-activated expression of the CAT-SYN A constructs under the control of HSV-1 promoters was significantly lower than that seen with the corresponding CAT plasmids (Fig. 1). These three promoters are generally induced to high levels by ICP4 and ICP0 (Everett 1984; O'Hare and Hayward 1985; Blair and Wagner 1986; Gelman and Silverstein 1986). The low induction seen here is likely the result of inefficient polyadenylation of CAT transcripts, as would be predicted by the lack of a G/U box (Gil and Proudfoot 1984; McDevitt et al. 1984). This was illustrated further with the plasmids containing the SV40 early promoter and enhancer, which is a very strong promoter compared with the HSV-1 promoters tested. Although the data are presented here relatively, as fold induction, the actual level of CAT activity seen with pSV2-CAT was consistently 20- to 50-fold higher than that found with pSV-SYN A. The level of expression obtained with these two plasmids was high enough to observe an effect by ICP27 on its own, without ICP4 or ICP0 (Fig. 1). Thus, pSV2-CAT expression was reduced to ~0.4-fold by ICP27 alone, whereas pSV-CAT-SYN A expression was stimulated ~5-fold. This suggests that ICP27 does not interact with ICP4 and ICP0 but that elevated expression of the targets, which is facilitated by these *trans*-activators, is required for the effects of ICP27 to be observed when weaker HSV-1 promoters are used. This was supported further in a related study on the effect of ICP27 on gene expression in a retroviral vector containing Moloney murine leukemia virus (MoMuLV) regulatory sequences, which again represents a strong promoter. ICP27 alone was shown to up-regulate expression (Chapman et al. 1992).

The previous result indicates that the activity of ICP27 on target gene expression is promoter independent. This was demonstrated further by the experiment shown in Table 1. In this experiment, the TK promoter was fused to the CAT plasmid derived from pSV2-CAT as described above, or to CAT-SYN A or LacZ. As stated earlier, neither an activation or repression of β-galactosidase activity was observed with ICP27 (Table 1, column 3), whereas pTK-CAT-activated expression was re-

Figure 1. Effect of ICP27 on CAT expression from different CAT target plasmids. HSV-1 promoters from the thymidine kinase gene (TK; early), the glycoprotein B gene (gB; leaky late), and the major virion capsid gene (VP5; leaky late), as well as the heterologous SV40 early promoter and enhancer region, were fused to two different CAT reporter constructs. The plasmids labeled CAT were derived from pSV2–CAT (Gorman et al. 1982) and contain the SV40 small t-antigen intron and early polyadenylation region 3' to the CAT-coding sequences as shown in the right panel. The constructs labeled CAT–SYN A were derived from pSV0d(ori[–])–CAT (Costa et al. 1985) and contain a synthetic oligonucleotide encoding the AATAAA poly(A) recognition site. Transfections were performed with target plasmids in the absence of effectors, and the values obtained were set at 1.0. Transfections with plasmids encoding the HSV-1 effectors ICP4 (pSG28K/B), ICP0 (pRS1), and ICP27 (pSG130B/S) were performed at equimolar concentrations of effector plasmids as described previously (Sekulovich et al. 1988). Results are presented as fold induction over the value obtained for the uninduced samples. Mean induction values are shown, and each transfection was performed at least six times to control for variability. The standard errors of the mean are shown as bars.

pressed (column 1). The expression of pTK–CAT–SYN A was activated further by ICP27, as was also seen with this plasmid in Figure 1. Even when targets that elicit a different response were cotransfected so that expression of different reporter genes could be measured, ICP27 affected expression of these targets as it does when they are transfected singly (Table 1). Thus, CAT expression was reduced 10-fold by ICP27, whereas β -galactosidase activity was unaffected when pTK–CAT and pTK–LacZ were cotransfected even though both targets contained identical promoter sequences and were present together in the same cells. Similarly, CAT activity was stimulated ~10-fold from pTK–CAT–SYN A, whereas β -galactosidase activity remained the same in the presence of ICP27. These results suggest strongly that ICP27 does not act at the transcriptional level, because its effects are promoter independent. They suggest further that ICP27 may have some involvement in mRNA processing or

stabilization because plasmids with different processing signals responded differently.

The effect of ICP27 on target gene expression is dependent on different mRNA processing signals

To examine the effect of ICP27 on gene expression in response to different mRNA processing sites in greater detail, a series of plasmids was constructed that differed only in processing signals. In all cases, HSV-1 TK promoter sequences from –510 to +55 (McKnight et al. 1981) were fused to CAT-coding sequences. The structure of each plasmid is shown in Figure 2. In the first five plasmids shown, different poly(A) regions were added 3' to the CAT-coding sequences. The first plasmid, termed pTK–CAT–SYN A, was described earlier. The plasmid termed pTK–CAT–SV40 EPA contains the early poly-

Figure 2. Effects of ICP27 on target gene expression depend on the presence of different mRNA processing signals. The target plasmids shown at *left* all contain the HSV-1 TK promoter from -510 to $+55$ and the CAT-gene coding sequences. The first five target plasmids contain different polyadenylation regions. pTK-CAT-SYN A was described in Fig. 1. The next four target plasmids contain the polyadenylation regions from the early region of SV40 (pTK-CAT-SV40-EPA), the late region of SV40 (pTK-CAT-SV40-LPA), the HSV-1 immediate-early ICP27 gene (pTK-CAT-HSV-IE PA), and the HSV-1 late glycoprotein C gene (pTK-CAT-HSV-LPA). The last four target plasmids contain introns, either the SV40 small t intron positioned 3' to the CAT gene (pTK-CAT-3'S-SV40-EPA and pTK-CAT-3'S-HSV-IE PA), or the SV40 late gene 16S/19S splice sites positioned in the 5'-leader region (pTK-CAT-5'S-SV40-EPA and pTK-CAT-5'S-SV40-LPA), as well as the designated polyadenylation regions. Target plasmids were transfected with the HSV-1 effectors ICP4 (pSG28K/B), ICP0 (pRS1), and ICP27 (pSG130B/S) as described previously (Sekulovich et al. 1988). The results are shown at *right* for each target plasmid and are presented as fold induction calculated relative to the value obtained with uninduced target plasmids, which was set at 1.0. (Hatched bar) 4 + 0; (solid bar) 4 + 0 + 27. Transfections were performed at least six times, and the standard errors of the mean are shown in parentheses.

denylation region from SV40, whereas pTK-CAT-SV40 LPA contains the SV40 late polyadenylation region. The plasmid pTK-CAT-HSV IE PA contains the 3'-untranslated region and poly(A) sequences from the ICP27 gene itself, which is an HSV-1 immediate-early gene, whereas pTK-CAT-HSV LPA contains the untranslated region and poly(A) site from the HSV-1 late gene encoding glycoprotein C.

In transfections with these plasmids (Fig. 2), CAT expression was activated by ICP4 and ICP0 at least fourfold above the uninduced level, except for pTK-CAT-SYN A, which was activated poorly as seen previously. When ICP27 was added to the transfections, a fourfold increase in CAT expression was found with pTK-CAT-SYN A and with pTK-CAT-HSV IE PA (Fig. 2). CAT expression was activated to high levels by ICP4 and ICP0 in transfections with pTK-CAT-HSV LPA (25-fold), and a slight further activation was seen with ICP27. No change in the level of CAT expression by ICP27 was found when either the SV40 early poly(A) site or late poly(A) site was present. Table 1 showed a similar result for the plasmid pTK-LacZ, which differed from pTK-CAT-SV40-EPA only in the reporter gene-coding sequences. This indicates further that responses seen with ICP27 are related

to RNA processing sites. In addition, because the targets used here differed only in the poly(A) regions, these data suggest that ICP27 may affect poly(A) site utilization. It has been reported that HSV-1 induces a processing factor that stimulates poly(A) site usage (McLauchlan et al. 1989).

We then analyzed the basis of the repressor effect of ICP27, which had thus far only been found using a target plasmid that contained the small t intron from SV40 positioned 3' to the CAT gene. Huang and Gorman (1990) reported that this intron, when present in CAT expression vectors, could lead to aberrant splicing. That is, cryptic 5' splice sites within the CAT-coding sequences were used sometimes, resulting in lower CAT expression compared with that seen in vectors lacking this intron. Although we had observed high levels of activation by ICP4 and ICP0 using CAT vectors derived from pSV2-CAT containing this intron (Fig. 1), it was necessary to determine whether the down-regulatory effect of ICP27 was related to the effects observed with this particular intron and its positioning. Therefore, we constructed three CAT plasmids with introns and compared these with the derivative of pSV2-CAT. In Figure 2, the construct formerly referred to as pTK-CAT is

called pTK-CAT-3'S-SV40 EPA to clarify the processing sites it contains and their positions. The plasmid designated as pTK-CAT-3'S-HSV IE PA was derived from the preceding plasmid, but the poly(A) region from ICP27 was substituted for the SV40 early poly(A) region. This was done to determine whether the poly(A) site made any difference in the level of expression in the presence of ICP27, because expression of pTK-CAT-HSV IE PA, which also contains this poly(A) site but no intron, was activated by ICP27. When the small t intron was present however, ICP27 repressed the ICP4- and ICP0-activated expression of pTK-3'S-CAT-HSV IE PA (Fig. 2).

To determine whether ICP27 could repress expression from other intron-containing plasmids without potential complications of aberrant splicing, the plasmids pTK-CAT-5'S-SV40-EPA and pTK-CAT-5'S-SV40 LPA were constructed. A fragment containing the SV40 19S late mRNA intron and a modified form of the SV40 16S late mRNA intron (Okayama and Berg 1983) was cloned into the 5'-leader region of pTK-CAT-SV40 EPA and pTK-CAT-SV40 LPA. CAT expression from both plasmids was activated efficiently by ICP4 and ICP0. CAT expression was reduced >10-fold when ICP27 was added. Therefore, the repressor effect of ICP27 appears to be related to the presence of introns rather than to a specific intron or to their positioning in target plasmids. In accord with this result, we found previously that ICP27 reduced ICP0 mRNA levels (Sekulovich et al. 1988). The ICP0 gene contains two introns within its coding sequences (Perry et al. 1986). Therefore, ICP27 appears to down-regulate expression of spliced mRNAs.

ICP27 affects the accumulation of CAT poly(A)⁺ mRNA in transfections with CAT expression plasmids containing different processing signals

The results presented in Figure 2 demonstrated that the activation effect of ICP27 correlated with the presence of different poly(A) regions and the repressor effect correlated with the presence of splice sites or introns. It was therefore predicted that the level of poly(A)⁺ CAT RNA would be increased by ICP27 when transfections were performed with a CAT-SYN A-derived vector, whereas CAT mRNA levels would be decreased using a CAT vector containing an intron. To test this prediction, cells were transfected with the vectors pSV-CAT-SYN A or pSV2-CAT, either alone or with ICP0- and ICP27-expressing plasmids. Total RNA was isolated and poly(A)⁺ RNA was selected by two successive purifications on oligo(dT)-cellulose columns. Poly(A)⁺ RNA was analyzed by ribonuclease protection assay (Fig. 3A). The structure of the CAT vectors used is shown in Figure 3B. Both target plasmids contained the SV40 early promoter and enhancer. This promoter was chosen to facilitate the RNA analysis because it is a stronger constitutive promoter than the HSV-1 promoters used elsewhere in this study. The 3' sequence of pSV-CAT-SYN A is also shown in Figure 3B. The synthetic oligonucleotide (Linney and Donerly 1983) beginning with the AATAAA

hexanucleotide is shown in uppercase letters. The adjoining vector region was sequenced and is shown in lowercase letters. The T- or GT-rich region, which has been termed the G/U box (Gil and Proudfoot 1984; McDevitt et al. 1984; McLauchlan et al. 1985) was not found in the usual position, 20–30 nucleotides downstream of the consensus hexanucleotide. However, a stretch of Ts can be found beginning ~40 nucleotides downstream from the AATAAA site.

The CAT antisense RNA probe that was used in the protection assays hybridized to a 250-nucleotide region starting at the 5' end of the CAT mRNA. This probe is described in the legend to Figure 4. In addition, a 205-nucleotide protected fragment was also seen, which most likely resulted from "breathing" or local denaturation of the CAT RNA hybrids during the assay. An A/T-rich stretch occurs within the CAT sequence. Cleavage in this region by RNase A would result in a protected fragment the size of the smaller fragment routinely observed.

The following observations were made in analyzing RNA levels in the experiment shown in Figure 3A, which support the hypothesis that ICP27 acts post-transcriptionally to affect RNA processing. First, as predicted by the results of the CAT assays, CAT mRNA levels were increased by ICP27 in transfections with pSV-CAT-SYN A (Fig. 3A) but were decreased by ICP27 in transfections with pSV2-CAT (Fig. 3B), despite identical heterologous promoter sequences in both target plasmids. For example, in cotransfections with ICP27 and ICP0, CAT poly(A)⁺ mRNA levels were increased 10-fold compared with the uninduced level and 5-fold compared with the ICP0-induced level for pSV-CAT-SYN A (Fig. 3A, lanes 1–3), whereas the amount of CAT poly(A)⁺ RNA seen in transfections with pSV2-CAT was decreased about fourfold when ICP27 was present compared with the level seen with ICP0 alone. This was in contrast to the effects seen with ICP0, a promiscuous transcriptional activator (Everett and Dunlop 1984; Everett 1987; O'Hare and Hayward 1987), which elevated CAT mRNA levels in transfections with both pSV-CAT-SYN A and pSV2-CAT (Fig. 3A). Second, the uninduced level of CAT poly(A)⁺ RNA detected in transfections with pSV-CAT-SYN A was ~20-fold lower than was found with pSV2-CAT, as would be expected if RNA transcribed from pSV-CAT-SYN A was not polyadenylated efficiently.

To investigate further the extent of polyadenylation of CAT RNA from transfections with pSV-CAT-SYN A, RNase protections were performed using a probe that spanned the 3' end of the CAT mRNA. The 490-nucleotide antisense RNA probe used spanned a region from within the CAT-coding sequences that was 220 bases upstream of the AATAAA hexanucleotide to a site 200 bases downstream of the AATAAA in the pBR322 vector portion of pSV-CAT-SYN A. The RNA probe also contained an additional 20 bases between the T7 transcriptional start site and the inserted CAT fragment and 30 additional bases from the site of the insertion in the polylinker to the *Hind*III site used to linearize the plas-

Figure 3. RNase protection analysis of polyadenylated RNA isolated from transfections with CAT vectors containing different processing sites. Total RNA was isolated from transfections with the target plasmids pSV-CAT-SYN A or pSV2-CAT, whose structures are shown in *B*. Transfections were performed either with the target plasmid alone (UN) or in the presence of plasmids expressing ICP0, ICP27, or the ICP27 activator mutant S23. Poly(A)⁺ RNA was selected by oligo(dT)-cellulose. RNase protections shown in *A* were performed with a ³²P-labeled antisense probe from the 5' region of the CAT gene. The probe and the expected 250-nucleotide CAT protected fragment are shown in Fig. 4B. Protections shown in *C* were performed with an antisense probe spanning the 3' region of the CAT mRNA. The probe and the expected poly(A)⁺ protection product are described in the text. To ensure that equal amounts of RNA were recovered and processed from each transfection, one-tenth of the RNA from each sample was hybridized with a probe specific for β -actin. The size of the actin protected product is 250 nucleotides. Size markers (shown in *C*) were obtained by end-labeling *Hinf*I-digested pBR322 DNA. The 3' RNA probe is also shown in *C*. Appropriate exposures of the autoradiographs were scanned by a laser densitometer to quantify the amount of CAT mRNA in each lane.

mid before transcription. Therefore, the RNA probe contained 50 bases of sequence that are not present in pSV-CAT-SYN A so that any undigested probe (490 bases) could be distinguished from CAT RNA that extended the length of the probe (440 bases).

Transfections were performed with pSV-CAT-SYN A; however, ICP0 was not added to the transfections. This was done to analyze the effect of ICP27 on polyadenylation directly without other *trans*-activators present because the CAT assay results in Figure 1 showed that activation could be seen with ICP27 alone. In addition, transfections were performed with an activator mutant

of ICP27. The S23 mutant contains a 2-amino-acid insertion within the ICP27 activation region (Hardwicke et al. 1989), as seen in Figure 5. Analysis of total RNA showed a predominant protected fragment of 440 nucleotides for the uninduced sample, as well as for RNA from transfections with ICP27 and mutant S23 (Fig. 3C, left). This indicates that the CAT RNA extended at least 200 bases past the AATAAA hexanucleotide. A second band of 240 nucleotides was also seen in the ICP27-induced sample (Fig. 3C, lane 2). The expected size of protected CAT poly(A)⁺ RNA is ~240 bases if cleavage and poly(A) addition occurred within 10–30 bases down-

Figure 4. RNase protection analysis of spliced CAT mRNAs in the presence or absence of ICP27. (A) Total RNA was isolated from transfections with the target plasmids pTk-CAT-5'S-SV40 LPA or pTk-CAT-3'S-SV40 EPA either uninduced (UN) or in the presence of plasmids expressing ICP4, ICP0, and ICP27. RNase protection analysis was performed using a 32 P-labeled CAT antisense RNA probe shown in B. In the analysis of RNA from transfections with pTk-CAT-5'S-SV40 LPA, a 375-nucleotide protected fragment was expected if the 19S splice acceptor was used, and a 310-nucleotide product was expected if the 16S splice acceptor was used. For RNA from transfections with pTk-CAT-3'S-SV40 EPA, a 250-nucleotide protected fragment was expected from the HindIII site at the start of the CAT mRNA to the EcoRI site within the CAT gene. In C, nuclear and cytoplasmic RNA fractions were isolated as described in Materials and methods from transfections with pTk-CAT-5'S-SV40 LPA, either uninduced or with plasmids expressing ICP4, ICP0, ICP27, or the ICP27 repressor mutant N2. Each fraction was hybridized with the CAT probe shown in B, and the protected products were analyzed on denaturing polyacrylamide-urea gels. An aliquot of each RNA sample from A and C was hybridized with a probe specific for β -actin to determine whether equal amounts of RNA were recovered and processed for each sample. Approximately one-eighth as much RNA was recovered in the nuclear fraction from the transfection with N2 as seen in C (lane 4). DNA size markers were obtained by end-labeling pBR322 DNA that had been digested with *HinfI*. Appropriate exposures of the autoradiographs were scanned with a laser densitometer to quantify the amount of protected product in each lane.

stream of the AATAAA site. This suggests that the 240-nucleotide band represented poly(A)⁺ RNA. The 240-nucleotide product was about one-third as abundant as the larger, full-length product in the sample with ICP27, as determined by densitometer scanning. The 240-base product could barely be detected in the uninduced sample (lane 1) and in the S23 sample (lane 3), being present in nearly 20-fold lower amounts than the 440-base product. To confirm that the 240-nucleotide product represented poly(A)⁺ RNA, a parallel sample to that shown in lane 2, where ICP27 was present, was fractionated on an oligo(dT)-cellulose column to enrich for poly(A)⁺ RNA. The material that bound to the column was designated

as poly(A)⁺, even though it was still contaminated with poly(A)⁻ RNA after just one fractionation. Material that flowed through the column was designated poly(A)⁻. The 240-nucleotide product was found only in the poly(A)⁺ fraction. In addition, the ratio of the 440-nucleotide product compared with the 240-nucleotide product was ~1.5 : 1, compared with 3 : 1 for total RNA. Also, twice as much of the 440-nucleotide product was found in the poly(A)⁻ fraction. These results indicate that the 440-nucleotide product represented unprocessed RNA. This was suggested further by Northern blot analysis of CAT total RNA from transfections with pSV-CAT-SYN A either uninduced or induced with ICP27. A

was similar in the nucleus and the cytoplasm. To address these points, transfections were performed with the plasmid pTK-5'-CAT-SV40 LPA, and RNA was isolated and analyzed by ribonuclease protection analysis. This plasmid was chosen because it contains the SV40 16S/19S splice sites placed 5' to the CAT-coding sequences rather than the SV40 small t intron 3' to the CAT gene, for which incorrect splicing patterns have been described (Huang and Gorman 1990). Control transfections were also performed with pTK-CAT-3'-SV40 EPA that contains the small t intron 3' to the CAT gene to directly compare the reduction in CAT mRNA accumulation.

The ³²P-labeled antisense RNA probe used in the protection assays is shown in Figure 4B. This 480-nucleotide RNA probe was transcribed in vitro from a pGEM-1-derived vector (see Materials and methods). It initiates within the CAT-coding sequences, spans the 16S/19S splice sequences, and terminates 10 bases past the *Bgl*III site, which is at position +55 in the TK regulatory sequences. The expected protected fragments include a 375-nucleotide fragment if splicing occurs at the 19S acceptor site, a 310-nucleotide fragment if splicing occurs at the 16S acceptor site, and a 45-nucleotide fragment from the untranslated leader region of TK to the splice donor site. This latter fragment was run off of the gels. In addition, we routinely observed a 260-nucleotide fragment that would result from the 16S spliced product if breathing occurred during the protection assays at the A/T-rich run within the CAT gene as was also observed in Figure 3. In protection assays using RNA from transfections with pTK-CAT-3'-SV40 EPA, a 250-nucleotide protected product was expected, as this vector does not contain the region between the *Bgl*III and the *Hind*III sites. This product is labeled CAT-protected fragment in Figure 4B. Again, as in Figure 3, a smaller fragment of 205 nucleotides was seen that likely results from breathing at the A/T-rich stretch in the CAT gene. In more recent experiments, the temperature of the RNase treatment has been reduced to 4°C, which results in a decrease in the amount of the smaller fragment, although it does not eliminate it (data not shown).

The overall amount of CAT RNA was reduced about sixfold in transfections with pTK-5'S-CAT-SV40 LPA and about ninefold for pTK-CAT-3'S-SV40 EPA in the presence of ICP27 compared with the level seen with ICP4 and ICP0 (Fig. 4A). These results are similar to what was found with pSV2-CAT (Fig. 3) and are in the range of the decrease in CAT activity found in transfections with these plasmids (Figs. 1 and 2). Therefore, the down-regulation of CAT RNA levels by ICP27 is not dependent on a specific intron or on the positioning of the intron. Furthermore, both the 19S spliced product and the 16S spliced product were found in transfections with pTK-5'S-CAT-SV40 LPA in the presence of ICP27, but their amounts were reduced relative to the level seen with ICP4 and ICP0 (Fig. 4A). In the latter case, splicing occurred about five times more frequently at the 16S acceptor site compared with the 19S site (Fig. 4A, lane 2). This result is in accord with that reported by Okayama and Berg (1983), who constructed this modified intron

and who found a similar ratio of 16S to 19S product. It can be concluded, therefore, that correctly spliced CAT mRNAs were produced in transfections with ICP27 but that their amounts were reduced.

It was also possible that ICP27 functioned at the level of mRNA transport similar to the HIV-1 *rev* protein (Chang and Sharp 1989; Emerman et al. 1989; Felber et al. 1989; Malim et al. 1989). If that were the case, then a difference in the distribution of spliced versus unspliced RNA between the nucleus and the cytoplasm would be expected. Until now, we had analyzed total RNA from transfected cells. Therefore, nuclear and cytoplasmic RNA fractions were prepared from transfections with pTK-5'S-CAT-SV40 LPA. We also included a transfection with the ICP27 repressor mutant N2 in this experiment. The N2 insertion mutation occurs within the repressor region of ICP27 (Hardwicke et al. 1989) as shown in Figure 5. As shown in Figure 4C, the distribution of the 16S and 19S spliced products was similar in the nuclear and cytoplasmic RNA fractions in all cases. Unspliced RNA could not be detected in the nuclear fraction from the transfection with ICP27 (lane 3), indicating that unspliced RNA did not accumulate but was degraded rapidly. The amount of spliced CAT RNA seen in transfections with ICP27 was about sevenfold lower than the amount seen with ICP4 and ICP0 (lane 2) in the nuclear fraction and sixfold lower in the cytoplasmic fraction. This result implies that ICP27 is not involved in facilitating RNA transport but exerts its effects on RNA levels in the nucleus. Consistent with the repressor mutant phenotype, as defined in CAT expression assays (Hardwicke et al. 1989), N2 did not lower the accumulation of spliced CAT RNA. The amount of spliced RNA detected in the nuclear fraction from the N2 transfection was only twice the level seen with wild-type ICP27 (cf. lanes 3 and 4); however, only about one-eighth as much nuclear RNA was recovered from this sample as determined by the actin control. In the cytoplasmic fraction, where recoveries were equivalent for all samples, there was four times as much 16S and 19S spliced product found in the N2 samples compared with the samples with wild-type ICP27, illustrating that this repressor mutant cannot down-regulate the accumulation of spliced RNAs.

ICP27 activator mutants can inhibit ICP4- and ICP0-induced CAT expression

Previously, we have reported the characterization of a series of in-frame insertion and deletion mutants within the ICP27 gene (Hardwicke et al. 1989). Mutations that mapped between amino acids 260 and 434 were termed activator mutants because they were defective in the ability to activate expression of pVP5-CAT, a derivative of the CAT-SYN A vector. Mutations within the carboxy-terminal 70–80 amino acids were also defective in the ability to repress the expression of pTK-CAT and were termed repressor mutants, although they were defective in both functions. To determine whether these mutants would act in a manner similar to what was

found with the CAT–SYN A- and pSV2–CAT-derived plasmids, an activator mutant and a repressor mutant were analyzed for their effects on the expression of target plasmids with different mRNA processing signals. The position of the activator mutant S23 and the repressor mutant N2 within the ICP27-coding region is shown in Figure 5.

Several of the target plasmids described in Figure 2 with different mRNA processing signals were used in transfections with either the wild-type ICP27 plasmid or with the activator or repressor mutants (Fig. 5). All of the target plasmids contained the TK promoter. The transfections were performed again with plasmids expressing the effectors ICP4 and ICP0 to activate the low constitutive level of expression. The effect of wild-type ICP27 or the mutants was compared with the activated response. In accord with the data in Figure 2, wild-type ICP27 repressed CAT expression from target plasmids containing introns (Fig. 5, reporter constructs 2, 3, 5, and 7) and activated CAT expression from pTK–CAT–SYN A and pTK–CAT–HSV IE PA, which contains the ICP27 polyadenylation region (Fig. 5). The repressor mutant N2 did not repress CAT expression from any of the targets with introns, nor did this mutant activate expression of any of the plasmids above the level seen with ICP4 and ICP0. Therefore, N2 behaved in a manner consistent with the previously defined phenotype in that it was defective in the ability to repress and to activate. The activator mutant S23 repressed the activated expression of the target plasmids with introns as efficiently as the wild-type ICP27 plasmid, and S23 failed to activate expression of pTK–CAT–SYN A in accord with the phenotype of activator mutants. However, an unexpected result was found in transfections with plasmids pTK–CAT–HSV IE PA, pTK–CAT–SV40 EPA, and pTK–CAT–SV40 LPA. S23 did not activate CAT expression above the ICP4- and ICP0-induced level, but the level of CAT expression was reduced 2.5- to 10-fold compared with the induced level. Target plasmids 2, 5, and 7 do not contain introns, and we had not observed previously any repressor effect by wild-type ICP27 on expression from targets without introns. Similar results were seen also with two other activator mutants, S13 and S1B (data not shown).

To investigate this observation further and to determine whether expression would be similar within the context of an HSV-1 infection, the experiments were repeated using wild-type and ICP27 mutant viruses. Transfections were performed with the same set of target plasmids, but no effector plasmids were added to the transfections. Instead, transfected cultures were infected with virus. Wild-type HSV-1 strain KOS infection provided ICP4, ICP0, and ICP27. The ICP27 null mutant 27-LacZ (Smith et al. 1992) is a viral insertion mutant in which the β -galactosidase gene was inserted in place of the ICP27-coding sequences. No ICP27 is expressed during infection of noncomplementing cells with 27-LacZ; therefore, this infection is equivalent to the condition of adding ICP4 and ICP0. Infections with the activator mutant S23 and the repressor mutant N2 were achieved by

infecting cell lines expressing these mutants with 27-LacZ. The *trans*-dominant cell line Sst26, which expresses S23, and cell line Nsi37, which expresses N2, have been described previously (Smith et al. 1991). The results shown in Figure 5 were consistent with the results obtained with transfections using effector plasmids for all of the targets tested. Thus, infection with KOS that expresses wild-type ICP27 resulted in activation of CAT expression with pSV–CAT–SYN A and pSV–CAT–HSV IE PA, and repression of CAT expression for targets with introns (plasmids 3, 4, 5, and 6). Infections of cell line Nsi37 yielded results similar to those seen in transfections with N2, namely neither repression nor activation was observed as predicted for a repression region mutant. Infection of the S23-expressing cell line also resulted in effects on CAT expression similar to what was seen in transfections using S23 plasmid. That is, repression of expression was seen with intron-containing targets but also, as with the S23 plasmid transfections, the induced expression of target plasmids 2, 5, and 7 by ICP4 and ICP0 (expressed in 27-LacZ infections) was also inhibited even though these targets do not contain introns. Therefore, the effects on CAT expression seen in the transfections using combinations of effector plasmids were not caused by nonspecific effects such as possible changes in the levels of the other activators by ICP27 or potential promoter competition as a result of cotransfecting a number of different plasmids. The same effects on expression were seen within the context of a viral infection. A possible explanation for the unexpected inhibitory action of S23 will be considered further below.

Discussion

Recent studies on the regulation of a number of cellular genes and on viral gene products have made it increasingly clear that post-transcriptional control mechanisms play a crucial role in modulating gene expression. In this study we have focused on the mode of action of ICP27, an essential regulatory protein of HSV-1, which is required for the switch from early to late gene expression during infection. By analyzing the effect of ICP27 on the expression of target genes during transfection experiments, it has been determined that ICP27 can act as a *trans*-activator or a *trans*-repressor of gene expression, depending on the target gene (Everett 1986; Block and Jordan 1988; Rice and Knipe 1988; Sekulovich et al. 1988; Hardwicke et al. 1989; Rice et al. 1989; McMahan and Schaffer 1990; Rice and Knipe 1990; Smith et al. 1991). During these studies we found that ICP27 affected the expression of two reporter genes differently, even though both contained identical promoter–regulatory regions. Two other human herpesvirus activator proteins have been reported to function in a reporter gene-dependent manner, specifically, BMLF1 from Epstein-Barr virus (Buisson et al. 1989; Kenney et al. 1989), a homolog of ICP27, and the major *trans*-activator protein from human herpesvirus 6 (Campbell et al. 1991). However, the basis for the reporter gene response was not investigated in these studies. In this report we analyzed the differ-

ences between the reporter genes and found that ICP27 activated or repressed expression of target plasmids in response to different mRNA processing signals.

The activation effect of ICP27 correlated with the presence of specific polyadenylation regions in the target genes (Figs. 3 and 4). McLauchlan et al. (1989) have reported that HSV-1 induces a processing factor that stimulates poly(A) site usage. In their studies, a late HSV-1 poly(A) site was processed more efficiently in nuclear extracts from HSV-1-infected cells compared with mock-infected cells, but another poly(A) site from an HSV-1 early gene was processed with equal efficiency. Although these investigators did not identify the factor, they provided evidence indicating that it was likely to be an immediate-early gene product. It is possible that ICP27 is this processing factor. In the target plasmid termed pSV-CAT-SYN A, CAT poly(A)⁺ RNA was barely detectable unless ICP27 was present in the transfection (Fig. 3). The polyadenylation signal in this plasmid lacks a sequence resembling a G/U box positioned 20–30 nucleotides downstream of the AATAAA hexanucleotide (Gil and Proudfoot 1984; McDevitt et al. 1984; McLauchlan et al. 1985). A small stretch of Ts is found starting ~42 nucleotides farther downstream. It has been shown that poly(A) site usage is decreased severely when the distance between the AATAAA signal and the G/U box is increased beyond 40 nucleotides (Gil and Proudfoot 1984; McDevitt et al. 1984; Brown et al. 1991). It is possible that ICP27 increases the amount of poly(A)⁺ RNA from this target by enhancing selection of this otherwise inefficient site. Poly(A)⁺ RNA of the size predicted by cleavage and poly(A) addition occurring 10–30 nucleotides downstream of the hexanucleotide was seen in significant amounts only when ICP27 was present (Fig. 3C). We have evidence that this downstream region must be present for increased expression by ICP27 because when the region 3' to the AATAAA sequence was deleted by treatment with Bal31, CAT expression could not be detected in the presence or absence of ICP27 (data not shown). In addition, in a related study, ICP27 was found to activate expression of a CAT vector containing MoMuLV regulatory sequences (Chapman et al. 1992). The stimulatory effect depended on the 3'-LTR sequences containing the polyadenylation region and was again promoter independent. This polyadenylation region has been reported to be inefficient when used in a luciferase expression vector compared with an SV40 poly(A) signal (Maxwell et al. 1991). These observations suggest that ICP27 somehow enhances polyadenylation efficiency from specific sites. These combined results suggest a role for ICP27 during polyadenylation.

A possible explanation for the inhibitory effect of the ICP27 activator mutant S23 on the induced expression of target plasmids without introns (Fig. 5) might be that the activation function occurs by direct participation of ICP27 in polyadenylation. It is possible that ICP27 interacts either with RNA or with processing factors to influence the efficiency of site selection. We have shown previously that activator mutants are *trans*-dominant and interfere with wild-type activity, whereas repressor

mutants are not (Smith et al. 1991). Activator mutants, therefore, might still bind or interact with the polyadenylation complex but would be unable to perform the wild-type selection or stimulation function and thus could interfere with normal processing. Clearly, to define the mechanism, biochemical evidence is required to identify the molecules with which ICP27 interacts. These studies are currently under way.

The repressor activity of ICP27 was linked to the presence of introns in the target genes (Figs. 3 and 5). When the SV40 small t-antigen intron was present 3' to the CAT-coding sequences or when SV40 16S/19S splice sites were present in the 5'-leader region of the CAT gene, significantly lower expression of CAT activity and decreased levels of CAT RNA were found. Although aberrant splicing patterns have been shown to occur with vectors containing the small t intron 3' to target-coding sequences (Huang and Gorman 1990), this is not likely to account for the effects observed with ICP27. In this study, expression of the vectors containing introns either 5' or 3' to the CAT gene was activated to high levels by the HSV-1 activators ICP4 and ICP0, and decreased expression was found only when ICP27 was present. Furthermore, correctly spliced products were found in the presence of ICP27 but in lower amounts (Fig. 4). The distribution of spliced products was similar in nuclear and cytoplasmic fractions, indicating that ICP27 exerted its effect on nuclear RNA. These results suggest that ICP27 may impair splicing. In this regard, HSV-1 infection results in the efficient shutoff of host protein synthesis. This is partly brought about by the action of the virion host shutoff function that facilitates the nonspecific degradation of host mRNAs and also viral RNAs (Read and Frenkel 1983; Oroskar and Read 1987; Strom and Frenkel 1987; Kwong et al. 1988). However, infections with ICP27 mutants are deficient in the shutoff of host protein synthesis (Sacks et al. 1985). One intriguing possibility to account for this observation is that ICP27 impairs splicing of host transcripts. HSV-1 is an unusual eukaryotic DNA virus in that the majority of its transcripts are not spliced (Wagner 1985). Those that are spliced are mostly immediate-early gene products that are made concomitantly with ICP27. In support of this hypothesis, Schroder et al. (1989) analyzed the change in processing of host cell transcripts in HSV-1-infected cells. They found that the processing efficiency of β -tubulin heterogeneous nuclear RNA (hnRNA) was reduced by 2 hr after infection and was inhibited almost completely by 5 hr, resulting in an accumulation of unspliced β -tubulin RNA. We have also found a decrease in spliced β -tubulin, actin, and GAPDH RNA after infection with wild-type HSV-1, but the level remained closer to that seen with mock-infected cells in ICP27 temperative-sensitive or null mutant infections (M.A. Hardwicke and R.M. Sandri-Goldin, in prep.). In addition, Martin et al. (1987) analyzed the distribution of host cell small nuclear ribonucleoprotein (snRNP) antigens after infection with HSV-1. A striking redistribution of snRNPs was seen. These investigators speculated that the formation of prominent snRNP clusters that condense in the nu-

cleus and then move to the periphery as infection proceeds results from partial inactivation of host pre-mRNA splicing as a result of HSV-1 infection.

The data presented here suggest that ICP27 participates in mRNA processing. We have considered other post-transcriptional control steps, but these seem to be less likely, although at this point they cannot be rigorously excluded. That is, ICP27 does not appear to be involved in transport because the distribution of spliced CAT transcripts was similar in the nuclear and cytoplasmic fractions. Furthermore, in studies using a monoclonal antibody against the pore-complex lamina fraction from CV-1 cells, Schroder et al. (1989) did not find any changes in the nucleocytoplasmic transport system after infection with HSV-1. RNA stability could also be affected by ICP27. We have not measured RNA half-lives directly to test this possibility. However, many mRNAs that exhibit regulated turnover contain sequences that affect their stability or additional factors or specific proteins are required for stabilization (Atwater et al. 1990). In the experiments described here, the mature CAT mRNA was the same in all cases, only sites that would affect pre-mRNA processing were different. Therefore, if ICP27 affected the stability of these CAT RNAs, it would have to have occurred before processing. On the basis of these considerations, it is more likely that ICP27 functions at the level of processing.

These findings are not only important from the point of view of gene regulation during viral infection but also because elucidating the nature of the interaction of ICP27 with components of the RNA processing machinery may help to uncover new information on polyadenylation and splicing reactions and how they might be linked.

Materials and methods

Recombinant plasmids, viruses, and cell lines

The effector plasmid pSG28K/B, which encodes the HSV-1 ICP4 gene; pRS-1, which encodes the ICP0 gene; and pSG130B/S, which encodes the ICP27 gene have been described previously (Sekulovich et al. 1988). The construction of the ICP27 insertion mutant plasmids S23 and N2 has been described previously (Hardwicke et al. 1989), as has the characterization of the S23-expressing cell line Sst26 and the N2-expressing cell line Nsi37 (Smith et al. 1991). The ICP27 viral insertion mutant termed 27-LacZ was described in Smith et al. (1992). pSV2-CAT was obtained from B. Howard (Gorman et al. 1982). pTK-CAT and pgB-CAT were derived from pSV2-CAT, as described previously (Sekulovich et al. 1988). The plasmid termed pVP5-CAT in Figure 1 is a derivative of pSV2-CAT. The promoter region from the HSV-1 VP5 gene from position -650 to -200 (Blair and Wagner 1986) was cloned into the *Hind*III site of pSV2-CAT after removal of the SV40 promoter sequences. The target plasmids designated as CAT-SYN A were derived from pSV0d(ori⁻)-CAT (Costa et al. 1985) obtained from E.K. Wagner. The HSV-1 TK promoter from -510 to +55 (pTK-CAT-SYN A), the glycoprotein B promoter from -520 to +40 (pgB-CAT-SYN A), the VP5 promoter from -650 to +200 (pVP5-CAT-SYN A), and the SV40 early promoter and enhancer sequences (pSV-CAT-SYN A) from pSV2-CAT were cloned

into the *Hind*III site of pSV0d(ori⁻)-CAT. The plasmid pTK-CAT-SYN A was used for the cloning of the other plasmids shown in Figure 2, which contain different polyadenylation sites. Polyadenylation regions were cloned into the *Bam*HI site that occurs ~90 bp downstream of the CAT translational stop codon. To obtain the plasmid termed pTK-CAT-SV40-EPA, a 133-bp *Hpa*I-*Bam*HI fragment, which contains the SV40 early poly(A) site, was added. Similarly, a 196-bp fragment containing the SV40 late poly(A) signal was obtained from the expression vector pTKβ (Clontech Laboratories) to construct the plasmid termed pTK-CAT-SV40 LPA. Two HSV-1 poly(A) sites were also used. The entire 3'-untranslated region was included in case there were sequences 5' to the poly(A) site that might be important. The poly(A) region from the immediate-early gene was derived from the ICP27 gene itself. A 497-bp fragment from a *Ssp*I site upstream of the ICP27 stop codon to a *Sst*II site ~300 nucleotides past the poly(A) site was purified from pSG130B/S. The CAT plasmid to which this fragment was added was termed pTK-CAT-HSV IE PA. The HSV-1 late poly(A) region was from the glycoprotein C gene that has an unusually long 3'-untranslated region (Frink et al. 1983). An 856-bp *Mse*I fragment originating 25 bp downstream of the translational stop and ending 15 bp downstream of the G/U box was used yielding the plasmid pTK-CAT-HSV LPA. The plasmid termed pTK-CAT-3'S-SV40 EPA in Figure 3 was called pTK-CAT in our previous studies (Sekulovich et al. 1988; Hardwicke et al. 1989; Smith et al. 1991) and is also termed pTK-CAT in Table 1 and Figure 2. pTK-CAT-3'-HSV IE PA contains the polyadenylation site from the ICP27 gene in place of the SV40 early poly(A) region in pTK-CAT-3'S-SV40 EPA. The SV40 16S/19S late gene splice sites positioned 5' to the CAT gene in pTK-CAT-5'S-SV40 EPA and pTK-CAT-5'S-SV40 LPA were obtained from pTKβ (Clontech Laboratories). A 180-bp fragment containing these sites was cloned into the *Bgl*II and *Hind*III sites of pTK-CAT-SV40 EPA and pTK-CAT-SV40 LPA. pTK-LacZ was derived from pON1 (Spaete and Mocarski 1985). The HSV-1 TK promoter was inserted upstream of the *lacZ* gene in pON1.

Cell culture and transfection

Rabbit skin fibroblast (RSF) cells were grown as described previously (Sekulovich et al. 1988). For experiments in which CAT activity or β-galactosidase activity was measured, transfections were performed in 35-mm-diam. six-well cluster dishes as described previously (Sekulovich et al. 1988). Cells were harvested 48 hr after transfection. CAT activity was measured by the diffusion assay of Neumann et al. (1987), modified as described previously (Sekulovich et al. 1988). β-Galactosidase activity was measured as described previously (Spaete and Mocarski 1985). In experiments in which viral infections were performed, RSF cells or the mutant-expressing Sst26 and Nsi37 cell lines (Smith et al. 1991) were transfected with the appropriate target plasmid. Sixteen hours later, cultures were infected with wild-type HSV-1 KOS or 27-LacZ (Smith et al. 1992). Cultures were harvested 12 hr after infection. Transfection experiments in which RNA was isolated were performed in 100-mm dishes as described by Sekulovich et al. (1988). RNA was harvested 24–36 hr after transfection.

RNA isolation and ribonuclease protection analysis

Total RNA was extracted by the guanidium thiocyanate method (Cathala et al. 1983). Poly(A)⁺ RNA was selected by using oligo(dT)-cellulose (Collaborative Research, Inc.) as described previously (Sandri-Goldin et al. 1983). Nuclear and cytoplasmic RNA fractions were isolated as follows. Cells were

scraped into ice-cold PBS, centrifuged at 3000g, and resuspended in a lysis buffer consisting of 10 mM Tris (pH 7.4), 3 mM CaCl₂, 2 mM MgCl₂, 0.5% NP-40, and 500 units of RNasin (Promega). The cells were lysed by five strokes of a Dounce homogenizer. The nuclei were pelleted by centrifugation at 2000g at 4°C. The supernatant containing the cytoplasmic fraction was removed, and an equal volume of 6.5 M guanidium thiocyanate was added to this fraction. The nuclei were washed gently in 1 ml of lysis buffer to remove any remaining cytoplasmic material and pelleted again. The nuclei were then suspended in guanidium buffer (3.7 M). Extractions with phenol–chloroform were performed as described previously [Sandri-Goldin et al. 1983]. 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 plasmid DNA had been removed from the samples before the RNase protection assays were performed.

A T7 expression plasmid was cloned in which a fragment from pTK–CAT–5'S–SV40 EPA was inserted into pGEM-1 (Promega) to allow the in vitro transcription of a probe antisense to the 5' region of the CAT gene. The 490-bp insert was from an *EcoRI* site 240 bp downstream of the CAT translational start to the *BglIII* site in the 5'-untranslated region of the TK regulatory sequences. The SV40 16S/19S splice sites are within this fragment as shown in Figure 4. To transcribe the antisense probe, the plasmid was linearized with *XbaI* whose recognition sequence occurs in the pGEM-1 polylinker. The 3' antisense probe was derived from a T7 expression plasmid containing a 450-bp *SspI* fragment from pSV–CAT–SYN A encompassing 220 nucleotides of CAT sequence, the polyadenylation site, and 200 bp of vector sequences downstream of the AATAAA. This fragment was inserted into the *SmaI* site of pGEM-1, and orientation was confirmed by DNA sequencing. The plasmid was cleaved with *HindIII*, whose recognition site occurs 30 bp downstream of the *SmaI* site in the polylinker. Antisense RNA was transcribed with T7 polymerase in the presence of [³²P]CTP. Equal amounts of the RNA samples (usually 50 µg) were mixed with 5 × 10⁵ cpm of antisense RNA, which had been gel purified. Hybridizations and RNase protection assays were performed essentially as described by Zinn et al. [1983]. The protected fragments were fractionated on 6% denaturing polyacrylamide–urea gels.

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