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Herpes Simplex Virus *trans*-Regulatory Protein ICP27 Stabilizes and Binds to 3′ Ends of Labile mRNA

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Previous work demonstrated that a herpes simplex virus type 1 (HSV-1) immediate-early function up-regulates beta interferon but not chloramphenicol acetyltransferase reporter genes driven by the strong simian virus 40 (SV40) or cytomegalovirus promoter-enhancer regions in both transient assays and stable cell lines. The different 3′ mRNA stabilization and RNA-processing signals from these two reporter genes appeared to be primarily responsible for this phenomenon. We now report that the HSV-1 ICP27 itself is sufficient to stimulate both steady-state accumulation and increased half-life of beta interferon reporter gene mRNA. Furthermore, the ability to respond directly to cotransfected ICP27 can be transferred to chloramphenicol acetyltransferase reporter genes by replacement of their SV40-derived splicing and poly(A) signals with the 3′ AU-rich and poly(A) RNA-processing signals from the normally highly labile beta interferon and c-*myc* mRNA species. ICP27 expressed in bacteria bound specifically to in vitro-generated RNA from both the beta interferon and c-*myc* intronless AU-rich 3′ RNA-processing regions, but not to the SV40-derived early-region splice signal and poly(A) sequences. By site-specific mutagenesis, we also show that individual ICP27 C-terminal amino acid residues that are positionally conserved in ICP27 homologs in other herpesviruses (D-357, E-358, H-479, C-400, C-483, and C-488) are critical for *trans*-regulatory activity. Importantly, several of these positions match mutations that are known to be essential for the role of ICP27 in the early-to-late switch during the virus lytic cycle. Therefore, our findings support the notion that HSV ICP27 modulates gene expression posttranscriptionally in part by targeting RNA.

During a lytic infection of susceptible cells by herpes simplex virus (HSV), the expression of viral genes occurs in a tightly regulated cascade fashion (reviewed in reference 55). Depending on the kinetics of expression and responses to inhibitors of macromolecular synthesis, the viral genes are classified into three broad classes: the immediate-early (IE) genes (α), the early genes (β), and the late genes (γ). The expression of IE genes occurs in the absence of any de novo protein synthesis and is induced by a virion-associated factor, VP16 (also known as α-TIF and Vmw65), which itself is encoded by a γ gene (2, 6). Four IE genes, namely, ICP0, ICP4, ICP22, and ICP27, encode proteins with regulatory functions that influence the expression of other HSV genes. However, despite their demonstrable regulatory properties, ICP0 and ICP22 are dispensable for growth of HSV in cell culture, whereas ICP4 and ICP27 are essential for virus growth (14, 15, 30, 48, 56).

ICP4 protein is a multifunctional protein of 175 kDa with intrinsic DNA-binding activity that down-regulates the expression of some IE and latency genes while augmenting the expression of both early and late viral genes (3, 16, 19, 41, 42, 49). Although the mechanism by which ICP4 activates early and late genes is not yet fully elucidated, it may depend on an ability to influence TATA-dependent assembly of general transcription factors via specific protein-protein contacts, together with some poorly defined interaction with the SpI transcription factor (20, 60).

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ICP27 (also known as IE63, IE2, and UL54) is a 63-kDa protein that is believed to be involved both in the switch from early to late gene expression and in augmenting viral DNA replication (12, 30, 34, 56). Functional analyses of HSV type 1 (HSV-1) mutant viruses with lesions in ICP27 suggest that ICP27 acts to down-regulate transcription of early genes and to up-regulate transcription of late genes. However, in transient expression assays ICP27 has been shown to act either as a repressor or as an activator in combination with ICP4 and ICP0, depending on the target gene examined (16, 22, 51, 58, 64). Nonetheless, the ability of ICP27 on its own to weakly stimulate the expression of the HSV glycoprotein B promoter does indicate that at least part of its positive regulatory effects may be independent of other viral regulatory proteins (52). More recent data implicate ICP27 as a novel viral regulatory protein with a potential role in 3′ mRNA and poly(A) processing, splicing, and mRNA stabilization events (7, 21, 23, 29, 32, 33, 46, 57, 61). In some studies, positive modulatory activity of ICP27 correlated with the presence of weak polyadenylation signals, whereas its negative modulatory activity was found to correlate with the presence of introns (57).

The specific function of ICP27 in the HSV replicative cycle is likely to be of central importance, since genes with homology to ICP27 have been described not only for other alphaherpesviruses but also for beta- and gammaherpesviruses. These include the IE gene BMLF1 of Epstein-Barr virus (EBV), the IE-52 gene of herpesvirus saimiri, the open reading frame 4 (ORF4) gene of varicella-zoster virus (VZV), the UL3 gene of equine herpesvirus 1 (EHV-1), and the UL69 gene of human cytomegalovirus (HCMV). All of the proteins encoded by these genes modulate gene expression. BMLF1 is a potent

activator of the chloramphenicol acetyltransferase (CAT) reporter gene and functions primarily at a posttranscriptional level, with its effects being reporter gene specific (4, 9, 10, 25, 27, 28, 68). In contrast, IE-52, ORF4, UL3, and UL69 are not yet known to mediate gene expression by posttranscriptional mechanisms, although they all upregulate CAT reporter genes (40, 44, 50, 63, 67, 70). Despite their apparent functional differences, one striking feature shared by ICP27 and its homologs in other herpesviruses is the presence of a partially conserved cysteine-rich motif in the C terminus of the polypeptide. Interestingly, both the positive and negative modulatory effects of ICP27 have been mapped to this cysteine-rich domain and conserved cysteine residues in the C terminus were shown recently to be critical for the *trans*-regulatory activities of both the VZV ORF4 and EHV-1 UL3 proteins (44, 63).

Previous studies by some of us have demonstrated that HSV encodes an IE function that leads to powerful stimulatory effects on the level of beta interferon (IFN-β) reporter gene mRNA in stable DNA-transfected cell lines (36). These effects appeared to involve mRNA stabilization and were not manifested by standard SV2CAT reporter genes or by IFN-β reporter genes containing the simian virus 40 (SV40) 3′ RNA-processing regions (37). We now report that this mRNA stabilization effect is reproduced by cotransfection with the HSV ICP27 gene in transient assays in the absence of other viral proteins and that the ability to respond to ICP27 upregulation can be transferred to CAT reporter genes containing the 3′ RNA-processing and mRNA instability signals from either the IFN-β or the c-*myc* gene. Furthermore, we show that ICP27 is an RNA-binding protein: it specifically binds to the intronless 3′ RNA-processing and poly(A) sequences derived from unstable transcripts such as IFN-β and c-*myc*. These findings indicate that ICP27 displays pleiotropic roles in modulating gene expression.

MATERIALS AND METHODS

Cell lines. The CD4-positive, human T-cell line A3.01 was obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (17). The A3.01 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine.

Plasmid constructions. The two IFN reporter plasmids (pJM40 and pJM41) containing versions of the SV2-IFN gene with different 3′ ends used in this study were described previously (37). SV2-IFN(40) contains the SV40 promoter-enhancer region driving human IFN-β_1 genomic DNA including the natural 3′ processing signals, whereas SV-IFN(41) contains a modified version of this with the 850-bp SV40 early-region poly(A) and 3′ signals replacing the IFN-β 3′ region. Plasmids pSVCATNP, pSVCATSV40, pSVCATβIFN, and pSVCATmyc have been described previously (44). In this panel of constructs, the reporter CAT gene is driven from the intact SV40 promoter-plus-enhancer region. In pSVCATNP, no eukaryotic RNA-processing signals 3′ to the terminator codon of the CAT gene are present. The plasmid pSVCATSV40 contains an 850-bp fragment with the SV40 early-region splice signals and poly(A) sequences 3′ to the CAT gene. Similarly, pSVCATβIFN and pSVCATmyc contain the 3′ untranslated region with the natural poly(A) signals from the human IFN-β and c-*myc* genes, respectively, placed immediately downstream from the CAT coding region.

To create a panel of CAT reporter genes driven from the strong, constitutive, major IE promoter (MIEP) of HCMV, with different 3′ RNA-processing signals, a 1.8-kb fragment containing the HCMV MIEP was first excised from the pG310 vector (45). This fragment, which contains the entire enhancer-promoter region as well as the untranslated exon 1 flanked by introns 1 and 2, was then used to replace the SV40 promoter segments in pSVCATNP, pSVCATSV40, pSVCATmyc, and pSVCATβIFN. The resultant plasmids were designated pCMVCATNP, pCMVCATSV40, and pCMVCATβIFN, respectively.

To express ICP27 under control of the HCMV MIEP, the coding region of ICP27 was excised from pGR215 (39) and cloned into the pG310 plasmid to generate pCMV27. A series of substitution mutants of ICP27 was then created by site-specific, oligonucleotide-directed mutagenesis with pCMV27 as the parent plasmid. All 12 cysteine residues in ICP27 were changed individually to serine residues, and the resultant plasmids were designated, by the nomenclature used previously for similar VZV ORF4 mutants (44), pCMV27C43S, pCMV27C115S, pCMV27C342S, pCMV27C363S, pCMV27C366S, pCMV27C400S, pCMV27C408S, pCMV27C414S, pCMV27C468S, pCMV27C483S, pCMV27C488S, and pCMV27C508S. In pCMV27H479P, codon 479, specifying histidine, was changed to a codon specifying proline. Codon 463, specifying tyrosine, was changed to a codon specifying either phenylalanine or serine, and the resultant plasmids were designated pCMV27Y463F and pCMV27Y463S. In addition, codon 357, specifying aspartic acid, was changed to a codon specifying asparagine and the resultant plasmid was designated pCMV27D357N. Finally, codon 358, specifying glutamic acid, was changed to a codon specifying glutamine and the resultant plasmid was designated pCMV27E358Q.

To express ICP27 as a glutathione *S*-transferase (GST) fusion protein in bacteria, the coding region of ICP27 was cloned into a modified pGEX-3 vector, pGH417 (11). In creating p27GST(10-512), a segment of ICP27 lacking only the first 9 amino acids was cloned in frame with the GST ORF.

To generate RNA segments representing the 3′ RNA-processing signals of SV40, c-*myc* and IFN-β genes, three plasmid constructs were created. In pBSSV40PA, an 840-bp *Xho*II fragment isolated from pSV2CAT carrying the SV40 early-region splice signals and poly(A) sequence was cloned into the pBS vector (Stratagene). pBSSV40PA was linearized with *Eco*RI prior to in vitro transcription with T3 RNA polymerase. Plasmid pJM52 contains a 617-bp fragment bearing the 3′ untranslated region of c-*myc* with its natural poly(A) site cloned into the pGEM I (Promega) vector. This plasmid was linearized with *Eco*RI prior to in vitro transcription with SP6 RNA polymerase. A 650-bp fragment containing the 3′ untranslated region with the natural poly(A) signal of human IFN-β was cloned into the pBS vector as a *Xho*I (blunted)-*Kpn*I fragment and designated pBSIFNPA. This plasmid was linearized with *Kpn*I and then treated with mung bean nuclease before being used for in vitro transcription with T3 RNA polymerase.

DNA transfections, CAT assays, and S1 hybrid protection assays. All DNA transfections and CAT assays with A3.01 cells were performed as previously described (45). Cellular mRNA from DNA-transfected Vero cells was prepared and assayed for IFN-β mRNA levels by annealing with ^{32}P-labelled riboprobes followed by S1 nuclease digestion and electrophoresis through 6% polyacrylamide–7 M urea gels as described previously (37).

In vitro transcription. To synthesize labelled RNA fragments, in vitro transcription reactions were performed in the presence of [α-^{32}P]UTP (200 μCi) by using a transcription kit (Stratagene) according to the manufacturer's instructions. Samples of linearized DNA (5 μg) were transcribed with the appropriate RNA polymerase (T3, T7, or SP6) in a 50-μl reaction volume for 30 min at 37°C and then subjected to treatment with DNase, phenol-chloroform extraction, ethanol precipitation, and final resuspension in 25 μl of RNase-free water.

Expression and purification of GST fusion proteins. *Escherichia coli* DH5α cells transformed with plasmids expressing GST fusion proteins were grown at 37°C with shaking overnight in 5 ml of Luria-Bertani (LB) medium supplemented with 200 μg of ampicillin per ml. The overnight culture was diluted 1:100 in fresh LB-ampicillin medium and grown an additional 2 h, after which protein expression was induced by the addition of 0.1 mM isopropylthio-β-D-galactoside (IPTG) for 6 to 8 h at 37°C. The bacterial cells were pelleted for 10 min at 5,000 rpm and resuspended in 2.5 ml of phosphate-buffered saline (PBS) containing 1% Triton X-100, TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone) (6 μg/ml), and phenylmethylsulfonyl fluoride (100 μg/ml). The bacterial cells were lysed by adding lysozyme (1 mg/ml) and incubating the mixture for 60 min on ice, and then they were subjected to freeze-thawing and sonication for 1 min in four 20-s bursts. After the cell suspension was centrifuged for 10 min at 12,000 × *g*, the supernatant was removed and saved. Three milliliters of 1.5% Sarkosyl–25 mM triethanolamine–1 mM EDTA solution was added to the cellular debris, and, following a 10-min incubation on ice and a 15-min centrifugation at 13,000 rpm, the supernatants were combined.

The GST and GST fusion proteins were purified by incubating the bacterial supernatants for 1 h with 1.33 ml of glutathione-Sepharose beads (Pharmacia) that had been washed three times and resuspended in PBS containing 1% Triton X-100. Following incubation, the beads were washed three times with PBS. The bound proteins were then eluted from the beads by adding 1 ml of 50 mM Tris supplemented with 10 mM glutathione and incubating the mixture at room temperature for 10 min with gentle rocking, followed by centrifugation. The eluted proteins were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stored at −80°C until needed.

In vitro RNA-protein interaction. To detect RNA-protein interactions, a Northwestern blotting procedure described by Chen et al. (8) was used, with minor modifications. Purified GST or GST-ICP27 fusion proteins were resolved on an SDS–10% polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schuell). The proteins were allowed to renature overnight by washing the membrane in binding buffer (10 mM Tris-HCl, pH 7.5; 50 mM NaCl; 1 mM EDTA; 1× Denhardt's solution) at 4°C. Following renaturation, the nitrocellulose membrane was incubated for 8 h at room temperature with 15 ml of binding buffer containing ^{32}P-labelled RNA probe. The membrane was then washed three times with binding buffer for 10 min, dried, and exposed to X-ray film.

RESULTS

Induction of IFN mRNA by cotransfection with HSV ICP27. Using Vero cell lines carrying a permanently integrated hybrid human IFN-β gene driven by strong constitutive promoters such as the SV40 or simian CMV MIEP, Mosca et al. (36) previously described a novel mechanism by which HSV-1 infection leads to a several-hundredfold increase in accumulation of IFN-β transcripts. The temperature-sensitive HSV-1 uncoating mutant *ts*B7 (1) failed to manifest this phenomenon at the nonpermissive temperature, indicating that VP16 (α-TIF or Vmw65), the late virion structural protein responsible for specific transactivation of HSV IE genes, was not sufficient. However, the HSV-1 *ts* K mutant (47), which produces a functionally inactive form of the ICP4 nuclear protein and no delayed-early or late gene products, was able to stimulate IFN-β accumulation, indicating that newly synthesized viral IE gene products other than ICP4 were required. This mRNA stabilization effect of HSV infection could also be generated in transient assays, but it did not occur with similar CAT reporter genes or with a version of the IFN-β gene in which the 3′ processing signals had been replaced with those from the CAT cassette (37).

To test whether either the ICP27 or the ICP0 gene product was capable of stimulating IFN mRNA accumulation, we cotransfected SV2-IFN target DNA with plasmids containing intact versions of these effector genes and examined their ability to alter IFN mRNA levels in transient expression assays. Treatment with the protein synthesis inhibitor cycloheximide (CHX) enhances the expression of IFN from the SV2-IFN target both transcriptionally and posttranscriptionally (37); hence, CHX treatment of transfected cells was included as a positive control. Assays for IFN-specific RNA by S1 riboprobe protection analysis (Fig. 1) detected little or no RNA in the samples after cotransfection either with pBR322 vector DNA (lane 1) or with the plasmid containing the intact HSV ICP0 effector gene (lane 4). In contrast, cotransfection of SV2-IFN target DNA with the HSV ICP27 effector plasmid led to greatly increased steady-state RNA levels (Fig. 1, lane 7). In all three samples, treatment of transfected cells receiving SV2-IFN target DNA with CHX for 4 h also resulted in high levels of IFN-β mRNA (Fig. 1, lanes 2, 5, and 8). Therefore, expression of the ICP27 protein alone is sufficient to account for the previously documented stimulation of these IFN-β reporter genes by HSV infection.

Stabilization of SV2-IFN mRNA by cotransfection with the ICP27 gene. An experiment designed to compare the time courses for degradation of IFN-β mRNA after CHX reversal treatment in the presence and absence of the coexpressed HSV ICP27 protein in a transient assay system is shown in Fig. 2. Since replacement of the 3′ untranslated region of the SV2-IFN(40) gene with 3′ sequences derived from the SV40 large-T antigen gene including the splice site and poly(A) signal results in stabilization of the normally labile IFN-β transcript (37), we included SV2-IFN(41), which contains these SV40-derived sequences downstream from the IFN-β coding sequence, as a constitutive control. A riboprobe spanning all of the SV40 early-promoter mRNA start sites was used in S1 analysis. The results confirmed that steady-state accumulation of SV2-IFN mRNA (in the absence of CHX) occurred both in the presence of the cotransfected HSV-encoded ICP27 gene product (but not in its absence) (Fig. 2, compare lanes 2 and 7) and when the 3′ region of the target SV2-IFN gene was replaced with SV40-derived 3′ sequences (lane 12). Furthermore, on the basis of the sizes of the protected 5′ mRNA species, the IFN-β mRNA produced was initiated at the expected multiple adjacent EE (early enhancer) mRNA start sites in the SV40 early promoter (nucleotide [nt] 320) and apparently also at the two nearby LE (late enhancer) start sites (nt 365). Note that both cotransfection of SV2-IFN(40) DNA with the HSV ICP27 gene and substitution with the SV40 3′ region in the SV2-IFN(41) construction gave multiple-start-site distribution patterns similar to those of CHX-induced SV2-IFN(40) mRNA. Therefore, the ICP27-mediated effect does not involve new initiation start sites or selectively alter the ratio of existing basal start sites. Importantly, maintenance of high steady-state levels of SV2-IFN(40) mRNA for greater than 4 h after CHX reversal was also accomplished in the presence of the HSV ICP27 gene product (Fig. 2, lanes 9 to 11), unlike the usual rapid shutoff and degradation (half-life of less than 1 h) which occur in the absence of ICP27 (Fig. 2, lanes 4 to 6). This result could be attributed either to stabilization of the SV2-IFN(40) mRNA by ICP27 or to new initiation events induced by preformed ICP27 during the reversal step (although the latter would depend on the ICP27 protein itself having a functional stability of greater than 4 h). However, either way, this accounts for the HSV induction and apparent stabilization being an IE function and is consistent with the notion that ICP27 may function posttranscriptionally with selectivity for labile mRNA.

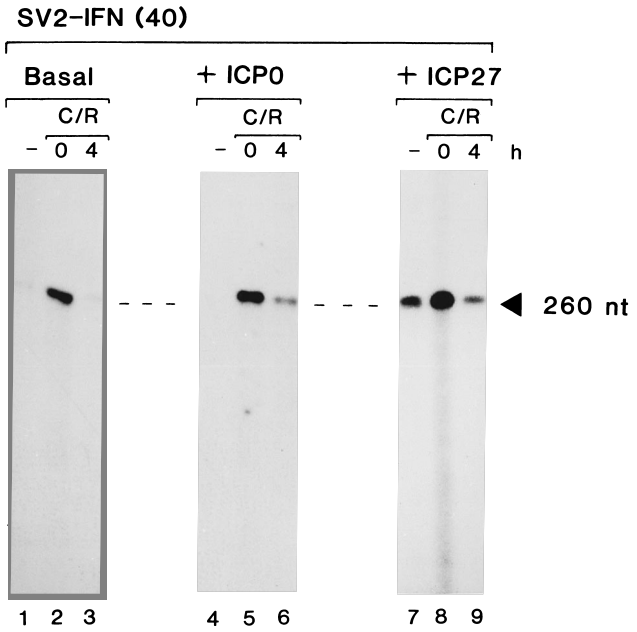

FIG. 1. S1 nuclease analysis of IFN mRNA from transiently cotransfected Vero cells receiving SV2-IFN(40) (pJM40) target DNA together with pBR322 (basal), ICP0 (pIGA15), or ICP27 (pGR215) effector DNA. Lanes for samples that were not treated with CHX at 48 h after DNA transfection are designated "−" (lanes 1, 4, and 7), and those for samples that were subjected to treatment with CHX (50 μg/ml) for 4 h plus reversal treatment for 0 or 4 h are designated "C/R" (lanes 2, 3, 5, 6, 8, and 9). Hybridization of total cell RNA to a ^{32}P-labelled IFN-β riboprobe (pJM94) gives a 260-nt protected mRNA species representing IFN-β sequences only (37).

The presence of 3′ RNA-processing signals from unstable mRNAs influences ICP27-mediated activation of CAT reporter genes. Having shown that HSV-induced enhancement of labile IFN-β transcripts was reproduced by cotransfection with the gene encoding ICP27, we next sought both to transfer the responsiveness to CAT reporter genes and to examine the contributions of different 3′ RNA-processing signals to ICP27-mediated effects in more detail. A panel of four target SV2-

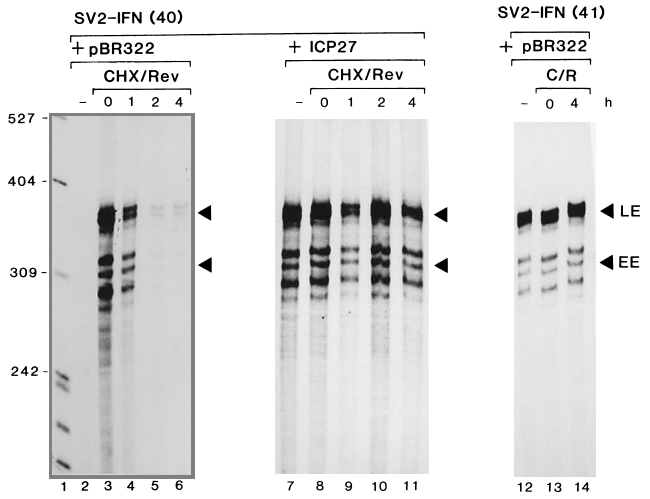

FIG. 2. Stabilization of SV2-IFN mRNA by cotransfection with the HSV ICP27 gene in transient expression assays. S1 nuclease protection analysis was performed to analyze the levels of SV2-IFN mRNA present at 48 h after DNA transfection of Vero cells with or without CHX treatment for 4 h and reversal for 0, 1, 2, or 4 h. Lane 1, single-stranded DNA size markers (ϕX174 DNA cleaved with *Msp*I); lanes 2 to 6, transfection of SV2-IFN(40) (pJM40) target DNA together with pBR322 DNA (basal); lanes 7 to 11, transfection of SV2-IFN(40) (pJM40) target DNA together with ICP27 (pGR215) effector DNA; lanes 12 to 14, control transfection of pSV2-IFN(41) (pJM41) target DNA together with pBR322 DNA (basal). In contrast to the riboprobe used for the experiment shown in Fig. 1, the riboprobe used in this experiment was synthesized from an SV2-IFN hybrid plasmid (pJM95a) and detects all SV40-derived transcription initiation sites. Numbers on the left indicate sizes in nucleotides. Labelling of lanes is as described in the legend to Fig. 1; CHX/Rev is the same as C/R.

CAT plasmids with identical promoter elements and reporter gene sequences but differing only in the 3′ RNA-processing signals was created (Fig. 3). A second, related set was also placed under the transcriptional control of HCMV MIEP regions instead of those from SV40. As shown in Fig. 4, the presence of SV40 poly(A) sequences, together with the splice signals from small-t antigen, resulted in high-level basal CAT

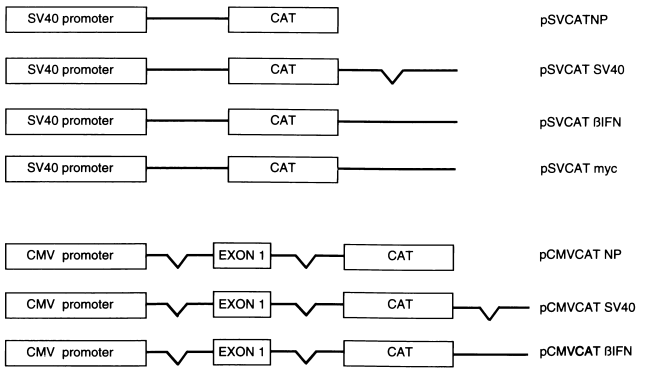

FIG. 3. Schematic representation of the target CAT constructs used. In the SV40 promoter-driven CAT reporter gene panel, pSVCATNP has no eukaryotic polyadenylation signals 3′ to the CAT coding region (the presence of cryptic poly(A) sites in the vector is, however, a possibility). In pSVCATSV40, an 850-bp fragment with the SV40 early-region splice signals and poly(A) sequences were present 3′ to the CAT gene. In pSVCATβIFN and pSVCATmyc, the 3′ untranslated regions containing the natural poly(A) signals and adjacent AU-rich blocks from the human IFN-β (650-bp) and c-*myc* (617-bp) genes, respectively, were placed immediately downstream of the CAT coding region (44). In the CMV panel, the CAT gene is driven by the HCMV IE promoter-enhancer region containing the untranslated exon I and exon II sequences of the major IE transcriptional unit.

activity in transient assays with A3.01 cells, regardless of whether the reporter gene was driven by the SV40 promoter or the HCMV MIEP. In contrast, the absence of any eukaryotic 3′ RNA-processing signals downstream from the CAT coding region resulted in very low-level basal activities.

Coexpression with ICP27 led to increased expression of CAT activity in all of the target-reporter genes, with the exception of the two versions bearing the 3′ RNA-processing signals from SV40. Surprisingly, in the presence of ICP27 the basal activity of pSVCATSV40 was lowered severalfold, although in pCMVCATSV40 there was no reduction of the basal activity.

To further explore the ability of SV40 poly(A) signals to negatively influence the effects of ICP27, we replaced the SV40 promoter in pSVCATSV40 with that of the HIV long terminal repeat (pHIVCATSV40), which did not result in any significant changes in the basal expression. Furthermore, in the presence of ICP27, as with pCMVCATSV40, the overall activity of pHIVCATSV40 was neither activated nor repressed (data not shown). Therefore, the presence of SV40 poly(A) signals and splice sites renders the transcripts refractory to the positive modulatory effects of ICP27, and with pSVCATSV40, the level of basal expression was negatively affected. It is important to note that the presence of an intron sequence in the transcript does not necessarily confer negative modulation, because all of the reporter genes in the HCMV MIEP-driven panel contain 5′-intron sequences yet all responded positively.

ICP27 binds to the 3′ RNA-processing sequences of unstable transcripts. From the experiments described above, it was clear that the 3′ RNA-processing sequences of a transcript alone can dramatically influence the outcome of cotransfection with ICP27. Posttranscriptional effects could potentially be mediated at many different steps, including elongation or processivity, efficiency of transport and poly(A) selection, or stability of the RNA substrate (reviewed in reference 43). Several viral regulatory proteins, for example, HIV tat and rev proteins, that are known to modulate gene expression posttranscriptionally have been shown to physically interact with their mRNA substrates (38). To explore the possibility that ICP27 may also interact with the RNA, an in vitro RNA-binding assay was performed. Bacterially expressed ICP27 as a GST fusion protein specifically bound to in vitro-synthesized ^{32}P-labelled riboprobes representing 3′ RNA-processing sequences from both the c-*myc* and the human IFN-β genes (617 and 650 nt, respectively) but not to the SV40 3′ RNA-processing sequence (840 nt) bearing the small-t antigen intron and the poly(A) signal (Fig. 5C) or to a riboprobe derived from the coding region of the CAT gene (data not shown). The relative purity of bacterially expressed GST fusion proteins used in the RNA-binding assays is illustrated in Fig. 5A. Figure 5B depicts the integrity of ^{32}P-labelled RNA probes used in the assays. These results indicate that ICP27 may be an RNA-binding protein that specifically interacts with the 3′ RNA-processing sequences of unstable transcripts and that this interaction occurs in the absence of any other accessory cellular or viral proteins. Furthermore, the binding of bacterially expressed protein to RNA implies that the binding is independent of complex posttranslational modifications of the ICP27 polypeptide.

Identification of functionally critical amino acid residues in ICP27. The HSV ICP27 polypeptide in the absence of any other effector proteins can modulate the expression of pSVCATSV40 in a weak negative fashion and that of pSVCATmyc in a strongly positive manner. To identify functionally important amino acid residues in the ICP27 polypeptide, single-substitution mutants were created by oligonucleotide-directed, site-specific mutagenesis. In order to focus on amino acids with potentially high infor-

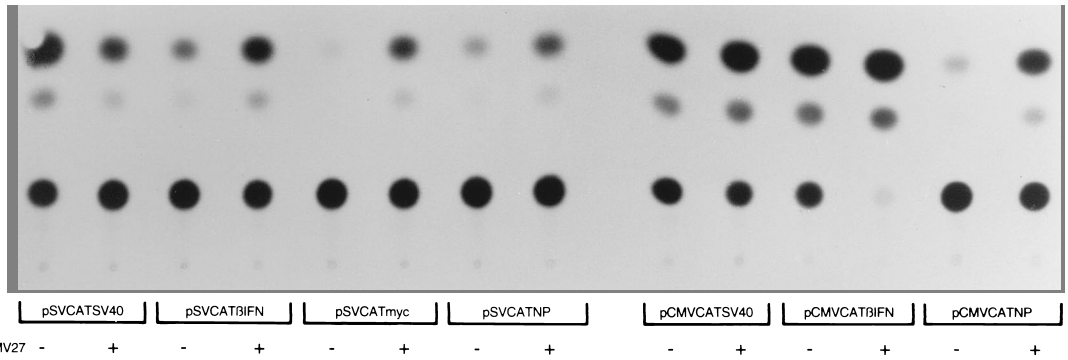

FIG. 4. ICP27 up-regulates the expression of CAT activity from reporter genes containing 3′ processing signals from unstable mRNAs. For cotransfection with the SV40 promoter-driven panel of target plasmids, 5 μg of target DNA was electroporated into A3.01 cells with 5 μg of pCMV27 plasmid DNA. Cells were harvested 48 h after DNA transfection, and the levels of CAT activity in the cell lysates were determined by using the same amount of total protein for all samples. CAT activity was quantitated with a PhosphorImager scanner and ImageQuant software (Molecular Dynamics). Because of the inherently higher-level activity of the HCMV promoter than of the SV40 promoter in A3.01 cells, both target and effector DNAs were used in smaller amounts (1 μg) in the cotransfection of the HCMV promoter-driven target panel. For each target plasmid, the basal activity and the activity in the presence of ICP27 were as follows: pSVCATSV40, 47% (basal) and 21.5% (ICP27 coexpression); pSVCATβIFN, 12% (basal) and 36% (ICP27 coexpression); pSVCATmyc, 2.7% (basal) and 19.3% (ICP27 coexpression); pSVCATNP, 6.3% (basal) and 16% (ICP27 coexpression); pCMVCATSV40, 53% (basal) and 74% (ICP27 coexpression); pCMVCATβIFN, 73% (basal) and 96% (ICP27 coexpression); pCMVCATNP, 6% (basal) and 36% (ICP27 coexpression).

mational content, we first compared the amino acid sequences of ICP27 homologs in VZV, EHV-1, bovine herpesvirus 1 (BHV-1), EBV, herpesvirus saimiri, and HCMV with that of ICP27 (Fig. 6). When these sequences were aligned, three distinct domains within the C-terminal half of each protein were found to have positionally conserved features. Within ICP27 (31), conserved region 1 (CR1) from amino acids 349 to 373 includes the totally conserved residues Asp-357 and Glu-358, CR2 from amino acids 377 to 402 includes the totally conserved Cys-400, and CR3 from amino acids 463 to 488 includes the totally conserved Tyr-463, Gly-466, His-479, Cys-483, and Lys-488. These conserved amino acids were then selected for the mutational analysis. With the objective of introducing minimal overall structural or conformational perturbations in the polypeptide, Cys residues were changed to Ser residues, Asp was changed to Asn, Glu was changed to Gln, Tyr was changed to either Ser or Trp, and His was changed to Pro.

In addition to the three conserved Cys residues, the remaining nine Cys residues in HSV-1 ICP27 were also changed to Ser residues individually because ICP27 has been reported to bind zinc (65) and Cys residues would be expected to play a role in the metal ion coordination used in oligomerization and protein-nucleic acid interactions. The substitution mutants were then cotransfected with the two target plasmids (pSVCATSV40 and pSVCATmyc) individually to assess the effects of the mutations.

Alterations in each of the three conserved cysteine residues, i.e., C-488, C-483, and C-400, completely abolished the functional activity of the polypeptide (Fig. 7). For the remaining nine cysteine residues, only C-508 substitution led to loss of function. Similarly, when the conserved H-479, D-357, and E-358 residues were changed to Pro, Asn, and Gln, respectively, complete ablation of ICP27 activity resulted. Interestingly, the mutation in which Y-463 was changed to F-463, thus retaining the hydrophobic aromatic core, was tolerated without any significant loss of activity. However, when Y-463 was substituted with S-463, the hydroxyl group being retained, a complete loss of function resulted. The steady-state levels of all nonfunctional mutant proteins were comparable to those of wild-type ICP27 protein as determined by immunoblot analysis with a polyclonal ICP27 antibody (data not shown). In addition, the mutations that ablated the activity of ICP27 do not map to the reported nuclear localization signals of ICP27 (35), thus making it less likely that the loss of activity is secondary to either defective nuclear localization or mutation-induced protein instability. It is important to note that with every single mutation that affected the activator function seen with the pSVCATmyc target, a parallel loss of repressor activity was seen with the pSVCATSV40 target, implying an intimate structure-function relationship between the two types of modulatory activity by ICP27.

DISCUSSION

The studies presented here describe for the first time a sensitive direct assay for transactivation by the HSV ICP27 protein in transient CAT assays. The only previous direct transactivation measured involved a small 3-fold stimulation of gB-CAT (51, 52, 54) whereas all other up-regulation effects involved 10-fold synergism with ICP4 and ICP0 on a cotransfected late VP5-CAT target, for example (16, 22, 52, 58, 64). In contrast, all other herpesvirus homologs of ICP27 have proved to directly up-regulate expression of standard CAT reporter cassettes (40, 44, 63, 67), but with the EBV and HSV versions this occurred in a reporter gene-specific manner such that Gal, β-globin, and growth hormone reporter genes driven by the same promoters failed to respond (4, 25, 27). In fact, in most cotransfection assays HSV promoter-containing CAT reporter genes with the standard SV40-derived 3′ splice signals (e.g., gD-CAT, TK-CAT, and ICP8-CAT) were repressed 5- to 10-fold in indirect assays in which ICP27 was cotransfected together with the ICP4 and ICP0 *trans*-activators (51, 58, 64). Our studies have also shown a small but specific direct repression of SV2-CAT. This *trans*-repression effect has been attributed to the presence of introns in most CAT cassettes, and more-recent studies have suggested that ICP27 can repress accumulation of spliced viral and cellular mRNA in infected cells to some degree (21, 23). However, complicated interactions between the IE proteins themselves may also have influenced the outcome of experiments with multiple *trans*-activators (39, 69, 71).

Note that the strong stimulation results with both IFN-β and CAT-IFN reporter genes (but not with standard spliced CAT cassettes) indicate that transactivation by HSV ICP27 is also

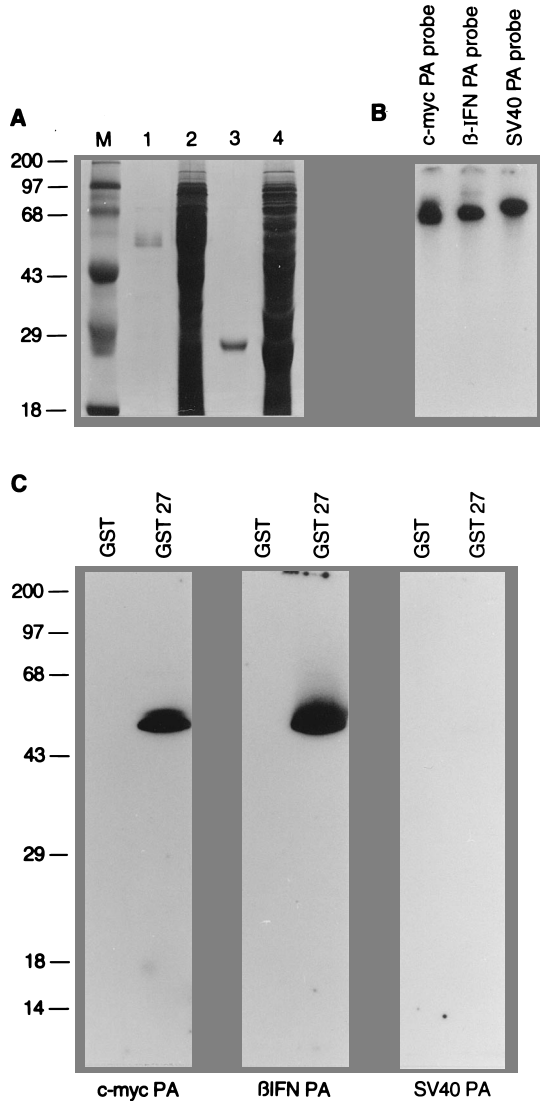

FIG. 5. The HSV ICP27 protein binds to the 3′ RNA-processing signals from unstable transcripts. (A) Bacterially expressed GST protein or GST-ICP27 fusion protein was purified by affinity chromatography with glutathione coupled to Sepharose (Pharmacia). Lanes 2 and 4 contain crude lysates from bacteria expressing the GST-ICP27 fusion protein and GST protein, respectively; lanes 1 and 3 contain affinity-purified GST-ICP27 fusion protein and GST protein, respectively; and lane M contains molecular weight marker proteins. (B) In vitro-generated riboprobes representing the 3′ RNA-processing signals of c-*myc* (617 nt), IFN-β (650 nt), and SV40 (840 nt) were electrophoresed on a 5% nondenaturing polyacrylamide gel to verify the integrity of these probes prior to their use in the RNA-binding assay. The amount of each probe loaded on the gel represents 1/20 of the total amount used in the RNA-binding assay. (C) GST and GST-ICP27 fusion protein (GST27) (40 μg of each protein) were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The membrane-bound proteins were renatured in situ and incubated with ^{32}P-labelled riboprobes (5×10^7 cpm) individually derived from c-*myc*, IFN-β, and SV40. PA, 3′ RNA-processing sequences. Numbers on the left indicate molecular weight (in thousands).

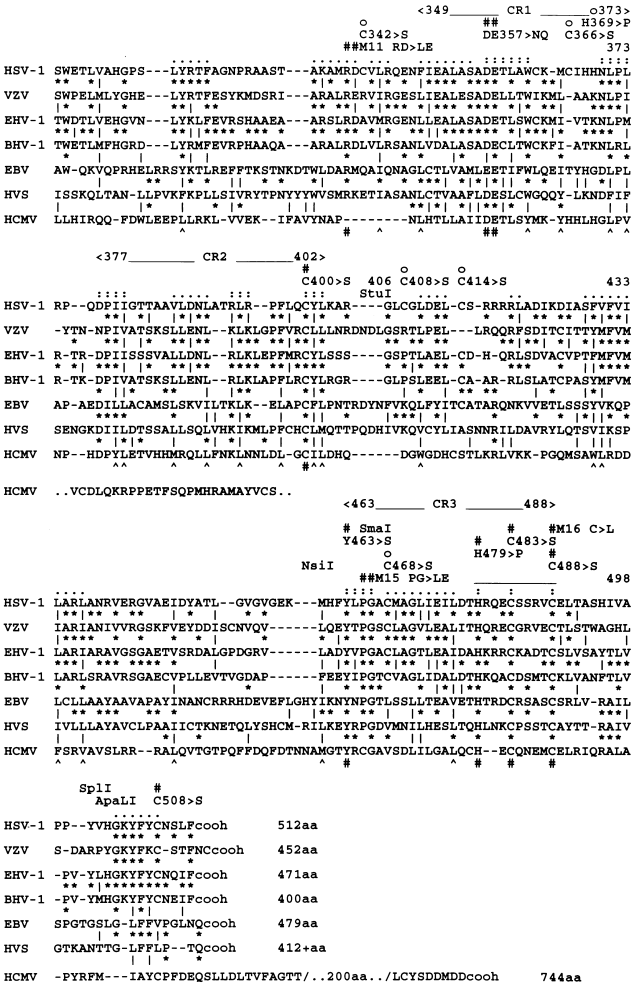

FIG. 6. Comparison of conserved motifs in the C-terminal region of ICP27 with ICP27 homologs in other herpesviruses and location of ICP27 point mutations. Note that no significant positional homology can be detected within the very hydrophilic N-terminal segments of these proteins. The single-letter amino acid sequence code is used for amino acids 311 to 512 from HSV-1 UL54, VZV ORF4, EHV-1 UL3, BHV-1 bie27 (18), herpesvirus saimiri (HVS) IE52, and HCMV UL69. The locations of CR1, CR2, and CR3 as defined here are indicated, together with the positions of all point mutations described here and reported by Rice and Lam (M11, M15, and M16) (53). Identity between positionally matching amino acids is indicated by * symbols, single dots indicate conserved motifs among alphaherpesviruses, double dots indicate motifs conserved among all seven herpesviruses compared, and similarity is indicated by | symbols, with the similar amino acid groupings being YWFLIVM, DE, RK, CH, and ST. Conserved hydrophobic positions are denoted by ∧ symbols. Mutated amino acid positions that have no effect on function are denoted by ○ symbols, whereas those that knock out functional activity are denoted by # symbols. aa, amino acids.

highly reporter gene specific but that it displays a target specificity different from that of the other herpesvirus ICP27 homologs. Most significantly, the powerful direct ICP27-mediated up-regulation targeted to the 3′ ends of unstable mRNAs has the same mutational sensitivity as that displayed by ICP27 for its essential role in the early-to-late switch in virus-infected cells (54). For example, the pattern of inactivation by amino acid point mutations at D-357 and E-358, C-400, Y-463, H-479, C-483, and C-488 in our transient assays correlates quite closely with the loss of functional activity by mutants M11(R340), M15(PG465/466), and M16(C488) described by Rice and Lam (53). These mutants are blocked in both IE mRNA shutoff and late-gene mRNA accumulation, although not in the ICP27-dependent stimulation of viral DNA synthesis. Note that mutations equivalent to C-400, H-479, C-483, and C-488 also destroy the direct transactivation of SV2-CAT by VZV ORF4 and the powerful activator domain function of a GAL4-ORF4 fusion protein targeted to a reporter gene with upstream GAL4-binding sites (5GE1bCAT) (44), implying

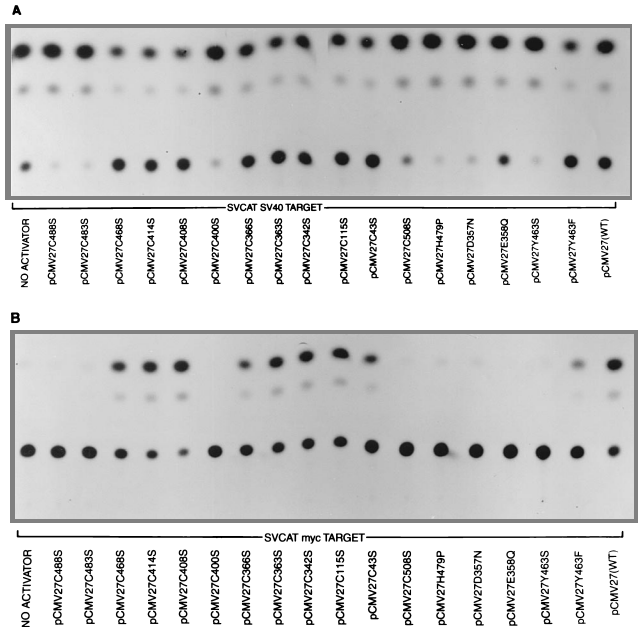

FIG. 7. (A) Identification of ICP27 mutants defective in basal-level repression of target constructs bearing SV40 3′ RNA-processing signals. A panel of ICP27 single-residue substitution mutants was created by site-specific, oligonucleotide-directed mutagenesis. Equimolar amounts (5 μg of each) of target (pSVCATSV40) and effector plasmid DNA were used in the cotransfection assays with A3.01 cells, and the level of CAT activity was determined as indicated in the legend to Fig. 4. The reduction in CAT activity in transfections with effector plasmids was calculated relative to the activity in the control experiment, in which 5 μg of pBluescript (Statagene) was used in cotransfection, and expressed as a percentage. The values for the various mutants are as follows (NR, no detectable repression): C488S, (C-to-S change at position 488), NR; C483S, NR; C468S, 64%; C414S, 64%; C408S, 62%; C400S, NR; C366S, 54%; C363S, 62%; C342S, 50%; C115S, 60%; C43S, 65%; C508S, NR; H479P, NR; D357N, NR; E358Q, 15%; Y463S, NR; Y463F, 63%; wild-type ICP27, 44%. (B) Identification of ICP27 mutants defective in activation of target reporter genes bearing c-*myc* 3′ RNA-processing signals. The panel of ICP27 single-residue substitution mutants described in the legend to panel A was cotransfected with the pSVC ATmyc target plasmid in A3.01 cells. Equimolar amounts (5 μg of each) of target and effector plasmid DNAs were used in the cotransfection assays with A3.01 cells, and the level of CAT activity was determined as indicated in the legend to Fig. 4. The fold induction of the CAT activity seen in transfections with effector plasmids was calculated relative to the level in the control experiment, in which 5 μg of pBluescript (Stratagene) was used in cotransfection, and the values for the various mutants are as follows (NA, no detectable activation): C488S, NA; C483S, NA; C468S, 13; C414S, 16; C408S, 19; C400S, NA; C366S, 9; C363S, 14; C342S, 13; C115S, 14; C43S, 8; C508S, NA; H479P, NA; D357N, NA; E358Q, NA; Y463S, NA; Y463F, 6; wild-type ICP27, 16.

that all of these activities may involve either a common activation mechanism or a common structural component of the protein.

Curiously, in our assays, the weak *trans*-repression by ICP27 on an SV2-CAT target containing the SV40-derived 3′-spliced signals also revealed the same pattern of inactivation as did the stimulatory activities, despite the inability of RNA from the 3′ end of the cassette to bind avidly to the GST-ICP27 protein. It should be emphasized, however, that similar mutational sensitivity does not necessarily reflect that a single function of the ICP27 polypeptide is responsible for both repressive and stimulatory activities. On the basis of the observations of Vaughan et al. (65) that the C-terminal Cys- and His-rich region of ICP27 binds zinc, but in a manner that is not dependent on the precise positioning of these residues, we suggest that the conserved nature and positioning of a subset of these residues (viz., C-400, H-479, C-483, and C-488) in all herpesvirus ICP27 homologs must play a key structural or functional role (irrespective of whether they participate in coordinating zinc).

There are considerable differences in the literature about the location of ICP27 domains involved in *trans*-repression. Our findings that direct repression of SV2-CAT was also abolished by almost the same set of mutations that knocked out 3′-end-mediated transactivation do not correlate well with the evidence given by McMahan and Schaffer (34) and Rice and Knipe (51), who argued that a protein containing only ICP27(1-406) still *trans*-repressed whereas N-terminal mutants were deficient in both *trans*-repression and DNA replication functions. Similarly, Rice and Lam (53) found that M11, M15, and M16 affected only synergistic *trans*-activation and not indirect *trans*-repression in cotransfection assays, whereas Rice et al. (54) found deficiencies in *trans*-repression with N-terminal region deletions. In contrast, Hardwicke et al. (22) reported that mutants with insertions between positions 262 and 406 lost transactivation ability but still repressed, whereas those with insertions between positions 434 and 512 lost both activities. Smith et al. (62) also dissociated *trans*-repression (still active) from transactivation (activity lost) in transient cotransfection assays with the temperature-sensitive mutant sLG4, whose mutation maps at codon 480; however, at the nonpermissive temperature this mutant virus was deficient in both IE mRNA shutoff and late mRNA accumulation. We do not have any explanation to resolve these discrepancies at present, and perhaps there are several different types of repression effects being measured.

The specific binding of bacterial GST-ICP27 to sequences at the 3′ ends of RNA from the three reporter cassettes that are most strongly transactivated by ICP27 cotransfection (namely, intact unspliced IFN-β and the CAT ORF with either c-*myc*- or IFN-β gene-derived 3′ regions) appears to provide a satisfying targeting mechanism that can lead to enhanced accumulation and increased half-life of the mRNAs produced. Although the 600- to 850-bp-long riboprobes used in this study are not strictly equivalent to the mature mRNAs expected to be produced by these reporter genes, they may in fact be even more appropriate targets as representations of primary transcripts that are not spliced (for the SV40 region) or polyadenylated, etc. We do not yet know whether the binding involves specific poly(A) signals or the long AU-rich instability-associated motifs present (24, 59, 66). Nonetheless, examination of ICP27 polypeptide sequences reveals the presence of two potential RNA recognition domains. The N-terminal cluster of arginines interspersed with glycines is related to a well-defined RNA-binding motif, RGG, present in heterogeneous nuclear ribonucleoprotein U, nucleolin, and EWS (Ewings sarcoma translocation gene) (5, 13, 26, 43). The repetitive RGGRRGR RRGRGRGG motif between amino acids 138 and 152 has recently been shown to target ICP27 to the nucleolus when not masked by adjacent segments of the intact protein (35). Potentially, this motif, which is not conserved in the other members of the ICP27 family (although they all have Arg-rich N-terminal regions), might act as a nonspecific RNA-binding core within a larger specific recognition domain. In addition, the putative Zn finger motif that has been described previously to be present in the C terminus of the polypeptide could also function as an RNA-binding motif (65). Mutational analysis of these potential RNA-binding motifs should help to clarify their role in RNA binding.

Although there is a correlation between the presence of an unstable 3′ RNA-processing sequence that binds ICP27 avidly and the level of positive modulation that is presumably related to stabilization, further study is needed to unravel the mechanistic aspects of this phenomenon. We cannot exclude the

possibility that the 3′ ends of these mRNAs provide an ICP27-mediated transcriptional initiation effect, but note that our previous nuclear run-on analysis supports the idea of the effect being posttranscriptional in HSV-infected cells (37).

A protein that binds to RNA can regulate the expression of a particular gene by a variety of mechanisms, such as facilitating or hindering the formation of specialized complexes at particular sites on the RNA by protein-protein interactions; they can also directly modify RNA structure either locally (modifying the conformation of bound RNA) or globally (modifying RNA secondary and tertiary structure). In addition, RNA-binding proteins can serve as structural components and form stable ribonucleoprotein particles that may serve to transport and localize RNAs.

The multiple functions of ICP27 clearly have far-reaching consequences for temporal expression of HSV genes, including the apparent repression of intron-containing IE transcripts while also stimulating the expression of late genes during the early-to-late switch in progression of lytic infection.

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