

Regulation of Herpes Simplex Virus Poly(A) Site Usage and the Action of Immediate-Early Protein IE63 in the Early-Late Switch

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The essential herpes simplex virus type 1 (HSV-1) immediate-early protein IE63 (ICP27) is pleiotropic in function, promoting the switch from the early to late phase of virus gene expression, and has effects on the posttranscriptional processes of mRNA splicing and 3' processing. We have investigated the role of IE63 in the regulation of viral mRNA 3' processing and of late gene expression. Our in vitro 3' processing studies demonstrated that HSV-1 infection induces an activity, which requires IE63 gene expression, responsible for an observed increase in 3' processing of selected HSV-1 poly(A) sites. Processing efficiencies at the poly(A) sites of two late genes, UL38 and UL44, shown to be inherently weak processing sites, were increased by the IE63-induced activity. In contrast, 3' processing at the poly(A) sites of selected immediate-early and early genes, stronger processing sites, was unaffected by IE63 expression. UV cross-linking experiments demonstrated that HSV infection caused enhanced binding of protein factors, including the 64-kDa component of cleavage stimulation factor (CstF), to poly(A) site RNAs from virus genes of all temporal classes and that this enhanced binding required expression of IE63. By immunofluorescence, the homogeneous pattern of the 64-kDa CstF protein distribution became slightly clumped with infection, whereas the splicing small nuclear ribonucleoprotein particles were reorganized into a highly punctate distribution away from the sites of virus transcription. This effect could create an increase in the relative concentration of 3' processing factors available to pre-mRNAs. Western blot (immunoblot) analysis showed that IE63 was required for expression of several true late genes and for the efficient and timely expression of the UL29 and UL42 early genes, integral components of the viral DNA synthesis machinery. Our data are consistent with two effects of IE63 on late gene regulation: firstly, a stimulation of pre-mRNA 3' processing and, secondly, as a requirement for expression of functions necessary for viral DNA synthesis.

During lytic infection, herpes simplex virus type 1 (HSV-1) gene expression occurs as a coordinately regulated cascade and three temporal classes of genes, immediate-early (IE), early, and late, can be differentiated according to the kinetics of their expression and requirements for ongoing DNA synthesis (1, 14, 49). Four IE proteins, expressed in the absence of de novo protein synthesis, regulate expression of early and late viral genes (reviewed in reference 38). Early proteins are involved in DNA metabolism and DNA replication, their expression signalling the onset of viral DNA synthesis. Expression of late viral genes can be divided into two phases on the basis of the stringency of their requirement for DNA synthesis (21, 47). The leaky late class of genes is expressed prior to DNA replication, but the true late genes are expressed only once viral DNA replication has commenced: inhibition of DNA synthesis reduces leaky late protein levels whereas true late proteins are hard to detect. The late proteins compose the majority of virus structural components.

The 63-kDa IE protein (IE63 or ICP27) encoded by the UL54 gene (26) is a nuclear phosphoprotein and is one of two IE proteins essential for lytic virus replication. IE63 plays a key role in the switch from early to late gene expression (37, 39), and this multifunctional protein has both *trans*-repressor and *trans*-activator functions (11, 30, 43, 44). Transfection studies have demonstrated that IE63 acts synergistically with IE pro-

teins IE175 and IE110 to repress expression from IE and early promoters and activate expression from late promoters (11, 30, 43); IE63 also affects the cellular localization of these two IE proteins (41, 44). At the posttranscriptional level of gene regulation, enhancement of gene expression correlates with the ability of IE63 to stimulate 3' processing (28, 29, 42) at certain poly(A) sites whereas the repressor function is associated with an inhibition of splicing. IE63 acts to down-regulate host cell gene expression most likely by inhibition of mRNA splicing, which has been observed both in vitro and in vivo (12, 35, 42). Martin et al. (22) have shown that HSV infection causes a redistribution of the cellular splicing small nuclear ribonucleoprotein particles (snRNPs) from a diffuse speckled pattern within the nucleus to a highly punctate distribution, and we have demonstrated that IE63 expression is necessary and sufficient for this effect (35). As few HSV genes are spliced, this could inhibit cellular RNA splicing by withdrawing the splicing snRNPs from active sites of transcription and pre-mRNA processing; this effect could augment the virion host shutoff activity (38).

IE63 affects viral DNA synthesis, as evidenced by IE63-deficient virus mutants which exhibit a range of defects, from making little or no DNA (23) to moderate or unaltered levels of DNA synthesis (39). In cells infected with an IE63 mutant virus, assembly of prereplicative complexes within the nucleus is defective, perhaps because of an observed conformational change and defective localization of the major DNA-binding protein (3). Following infection with an IE63 mutant virus, Uprichard and Knipe found an underexpression of several proteins required for virus DNA replication (46), implying that

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the reduction in viral DNA synthesis observed was due to reduced expression of essential replication genes (46).

Here, we examine further the role of IE63 in the regulation of mRNA 3' processing and in the switch from early to late gene expression. A previously identified activity induced in HSV-infected cells, active both *in vitro* and *in vivo*, selectively increased 3' processing of an HSV-2 late poly(A) site but had no effect on a second poly(A) site common to genes from the IE, early, and late temporal gene classes (29). The activity, present from early stages of virus infection and termed LPF, required expression of IE63 protein (28). There are several well-documented examples in both mammalian and viral systems which employ alternative or regulated usage of poly(A) sites as a means of controlling gene expression (4, 5, 9, 16, 20, 34, 51). Broadly, poly(A) site usage is dependent on inherent poly(A) site efficiency and/or on the concentrations of components of the 3' processing complex.

Here we have extended our *in vitro* polyadenylation studies, to demonstrate that this IE63-dependent activity selectively increases 3' processing at poly(A) sites of two late HSV-1 genes, UL38 and UL44, while having no effect on 3' processing of four additional poly(A) sites from genes of the IE and early temporal classes. Strikingly, the late poly(A) sites which responded to the IE63-induced activity were inherently less efficient than the IE and early poly(A) sites, which were unresponsive; RNA comparisons did not identify responder sequences. We propose that temporal expression of these late HSV-1 genes may be influenced by their poly(A) site strengths.

The complexes present in mammalian cell extracts which are required for 3' processing of mRNAs are formed by the functional association of two major groups of proteins. The first group, termed cleavage and polyadenylation specificity factor (CPSF), consists of four proteins which bind to the poly(A) signal AAUAAA (7, 15, 48). The second group, termed cleavage stimulation factor (CstF), is a complex of three proteins which serves to stabilize the CPSF-pre-mRNA complex and is required for efficient polyadenylation (7, 48, 50, 54). UV cross-linking analysis shows that 160- and 35-kDa CPSF components and a 64-kDa CstF component bind directly to pre-mRNA substrates (8, 15, 45, 52); binding of the 64-kDa component requires a U-rich tract 3' to the poly(A) signal (19, 52).

The UV cross-linking studies presented here show that HSV-1 infection causes a marked increase in binding of nuclear proteins to pre-mRNAs from HSV-1 poly(A) sites of all temporal classes; further, this increased binding requires expression of IE63 protein. The UV cross-linked proteins were of a size comparable with that of known components of 3' processing complexes, and one band was identified as the 64-kDa component of CstF. This increase in binding of nuclear factors could produce a concomitant increase in mRNA 3' processing.

Our indirect immunofluorescence experiments demonstrate that the homogeneous pattern of distribution of the 64-kDa CstF component is similar in infected and uninfected cells whereas in infected cells the splicing snRNPs are present in a highly punctate distribution. Thus, the polyadenylation factors, present at the sites of virus transcription, are spatially separated from the splicing factors. This effect could lead to an apparent increase in the concentration of polyadenylation factors available to pre-mRNAs and to an overall increase in mRNA 3' processing.

Here we demonstrate that Western blot (immunoblot) analysis of viral protein expression in cells infected with an HSV-1 IE63 null virus mutant in comparison with wild-type (wt) infections demonstrated that IE63 gene expression was required for production of proteins from three late genes and for the efficient expression of two early genes required for virus DNA

synthesis. This represents a further effect of IE63 on late gene expression, which acts by an indirect effect on viral DNA synthesis.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown as monolayers in Dulbecco's medium supplemented with 5% newborn calf serum and 5% fetal calf serum. Vero and Vero 2.2 cells were maintained in Glasgow minimum essential medium supplemented with 5% newborn calf serum and 5% fetal calf serum. BHK cells were grown in Glasgow minimum essential medium supplemented with 10% newborn calf serum. Stocks of wt HSV-1 strain 17⁺ and HSV-1 strain KOS (the parental strain of virus 27-LacZ) were grown at 37°C on BHK monolayers. Virus 27-LacZ, a gift from R. M. Sandri-Goldin, in which the IE63 gene is inactivated by insertion of a LacZ cassette, was grown on the complementing cell line, Vero 2.2 (44).

Plasmids. Construction of the control plasmid pSAU5 containing the tandemly arranged IE and L poly(A) sites has been described previously (29). Plasmids containing the selected HSV-1 poly(A) site fragments spanning the region 200 bp upstream and 200 bp downstream of the poly(A) signal were constructed by PCR and inserted into the multicloning site of pGEM-1.

***In vitro* polyadenylation reaction.** HeLa cell monolayers (6×10^7 cells) were infected with HSV-1 strain 17⁺ at a multiplicity of 10 and left for 8 h (time postinfection commences as soon as the virus is added to the cells). Nuclear extracts were prepared by a small-scale extraction method as described previously (17, 39). Precursor RNA was transcribed from the SP6 promoter of the pGEM-1 plasmid series containing poly(A) site sequences from HSV-1 genes IE110, IE63, UL29, UL23, UL38, and UL44 and labelled with [α -³²P]UTP, as described previously (29). *In vitro* polyadenylation reactions were carried out with 1×10^4 to 2×10^4 cpm of pre-mRNA in 1 mM 3' dATP-5 mM creatine phosphate-2.5 mM Tris-HCl (pH 7.6)-2.5% polyethylene glycol at 30°C for 2 h (29). Reaction products were separated by electrophoresis on 6% polyacrylamide gels, and the bands of radiolabelled processed and unprocessed RNA were visualized by autoradiography. The relative quantities of processed and unprocessed radiolabelled RNA were determined with a Molecular Dynamics PhosphorImager, and processing efficiencies were calculated as a percentage of the amount of cleaved product relative to total input RNA.

Western blot analysis. Protein extracts were prepared from wt HSV-1 KOS and 27-LacZ-infected Vero cells and 27-LacZ-infected Vero 2.2 cells. Infections were carried out over a 24-h time course, and cells were harvested at 2, 4, 8, 12, 18, and 24 h postinfection. Viral and cellular proteins were extracted from the cells by lysis and separated under denaturing conditions on sodium dodecyl sulfate (SDS) polyacrylamide gels. Following transfer to nitrocellulose membranes, the viral proteins were visualized with antibodies described below and the enhanced chemiluminescence (ECL) detection system (Amersham, Little Chalfont, United Kingdom).

Antibodies. All antibodies were diluted in Tris-buffered saline containing 0.05% Tween 20. Proteins encoded by the IE110, UL29, US6, and US8 genes were detected with mouse monoclonal antibodies at a dilution of 1:100, and the UL42 protein was detected with mouse monoclonal antibody at a dilution of 1:500 (these antibodies were provided by A. Cross, Institute of Virology, Glasgow, Scotland). Proteins encoded by the US11, UL45, and IE63 genes were detected with rabbit polyclonal antibodies at the following dilutions: anti-US11 [14473/4], 1:20; anti-UL45 [72], 1:10; and anti-IE63, 1:100 (these antibodies were provided by H. S. Marsden, Institute of Virology, Glasgow). The UL38 protein was detected with rabbit polyclonal antipeptide antibody at a dilution of 1:200 (a gift from W. S. M. Wold, Institute of Molecular Virology, St. Louis University Medical Center [53]). The UL44 protein was detected with rabbit polyclonal antibody R47 at a dilution of 1:100 (kindly provided by G. H. Cohen and R. J. Eisenberg, Department of Microbiology, University of Pennsylvania). The cellular 64K protein component of the CstF complex was detected with a monoclonal antibody at a dilution of 1:100 for both Western blot and immunofluorescence (kind gift from C. C. MacDonald, Department of Molecular Biology, Howard Hughes Medical Institute, Princeton, N.J. [19]). The anti-U2 B' antibody (Euro-Diagnostica) was used at a dilution of 1:10 for immunofluorescence.

UV cross-linking analysis of RNA-protein interactions. Mock-infected and HSV-1 strain 17⁺-infected nuclear extracts prepared as for the *in vitro* polyadenylation assays were dialyzed for 3 h against binding buffer (60 mM KCl, 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 8], 1 mM MgCl₂, 10% glycerol) at 4°C before use. RNA labelled with [³²P]UTP was transcribed from the poly(A) site-containing plasmids, as described for the *in vitro* polyadenylation reactions, and was resuspended in binding buffer at 300 cps. One microliter of nuclear extract (10 to 15 µg of protein) was incubated with 1 ml of ³²P-labelled RNA in a total volume of 10 µl of binding buffer for 30 min at room temperature. The reaction mixtures were cross-linked with 254-nm UV irradiation with a Stratilinker (energy = 250 mJ/cm²). RNase A was added to a final concentration of 1 mg/ml, and the mixture was incubated for 15 min at 37°C. An equal volume of loading buffer was added, and the samples were heated to 100°C for 5 min before electrophoresis on SDS-10% polyacrylamide gels under reducing conditions. ³²P-labelled proteins were visualized with a Molecular Dynamics PhosphorImager.

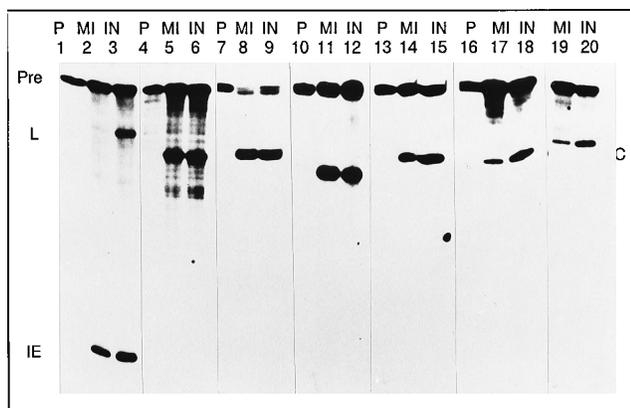


FIG. 1. The effects of HSV-1 infection on 3' processing of selected HSV-1 poly(A) sites. RNA processing reaction products from cleavage of pre-mRNA with mock-infected (MI) and HSV-1-infected (IN) HeLa cell nuclear extracts are shown. P is unprocessed precursor RNA. Lanes 1 to 3, control plasmid pSAU5; lanes 4 to 6, IE63 gene poly(A) site; lanes 7 to 9, IE110 gene poly(A) site; lanes 10 to 12, UL29 gene poly(A) site; lanes 13 to 15, UL23 gene poly(A) site; lanes 16 to 18, UL38 gene poly(A) site; lanes 19 to 20, UL44 gene poly(A) site. Pre is unprocessed precursor RNA; IE and L are RNA products resulting from cleavage at the IE and L poly(A) sites of plasmid pSAU5, respectively; C represents RNA species (200 to 250 nucleotides) generated by 3' processing at the test poly(A) sites.

Indirect immunofluorescence. Immunofluorescence experiments were performed as described by Phelan et al. (35).

RESULTS

Upregulation of 3' processing efficiencies at the poly(A) sites of late HSV-1 genes. An HSV-induced activity, detected from IE times postinfection, selectively increased the 3' processing efficiency in vitro of the UL38 HSV-2 late gene poly(A) site (termed L site) and had no effect on processing of the poly(A) site common to the US10, US11, and US12 genes of the early, late, and IE temporal classes, respectively (termed the IE site) (29). As the range and specificity of the activity were not known, the in vitro 3' processing assay (29) was repeated with a range of selected HSV-1 poly(A) sites from genes representing the different temporal classes. PCR-amplified sequences from the 3' regions of the IE genes IE63 and IE110, the early genes UL29 and UL23, and the late genes UL38 and UL44 were cloned separately into pGEM-1 vectors. These segments contained sequences 200 bp upstream and 200 bp downstream of the poly(A) signal AAUAAA. Nuclear extracts from mock-infected and HSV-1-infected HeLa cells were used to process precursor mRNAs synthesized from these plasmids. Pre-mRNA from the plasmid pSAU5 (29), which contains the tandemly arranged HSV-2 IE and L poly(A) sites, was included as a control.

With mock-infected nuclear extracts, the IE poly(A) site of pSAU5 was processed efficiently and the L poly(A) site was processed weakly (Fig. 1, lane 2). In contrast, both the IE and L poly(A) sites were processed efficiently when HSV-1-infected nuclear extracts were used (Fig. 1, lane 3). The in vitro 3' processing efficiencies of the late gene UL38 and UL44 poly(A) sites were increased threefold when infected nuclear extracts were used (Fig. 1, compare lanes 17 and 18 and lanes 19 and 20). However, the HSV-1 poly(A) sites of the IE genes IE63 and IE110 and the early gene UL29 showed no discernible change in processing efficiencies when either mock-infected or HSV-1-infected nuclear extracts were used to process these sites (Fig. 1, lanes 4 to 12). The UL23 poly(A) site (Fig.

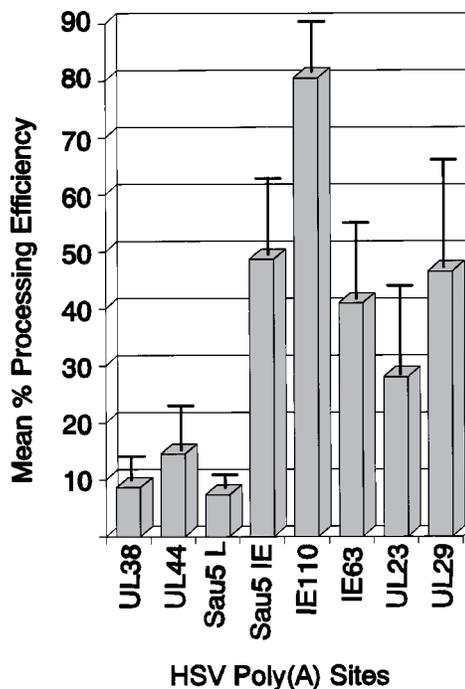


FIG. 2. Basal levels of 3' processing efficiency of the HSV-1 test poly(A) sites (UL38, UL44, IE110, IE63, UL23, and UL29), and of the IE and L sites on plasmid pSAU5. Precursor mRNAs were processed with mock-infected HeLa cell nuclear extracts. The percent processing efficiency is expressed as the mean of a minimum of six individual experiments using different nuclear extracts. Error bars show the standard deviation around the mean.

1, lanes 13 to 15) was somewhat variable in response but showed no consistent increased usage with infected nuclear extracts.

The HSV poly(A) sites which exhibit an inducible 3' processing activity are inherently weak. With mock-infected nuclear extracts, quantitative analysis of the in vitro 3' processing efficiencies of the six test poly(A) sites revealed striking differences in their basal levels of processing efficiency. The mean percent processing efficiencies of the test poly(A) sites are shown in Fig. 2; efficiencies of the UL38, UL44, and pSAU5 L sites were two- to fivefold lower than those of the IE110, IE63, UL23, UL29, and pSAU5 IE poly(A) sites. Thus, the poly(A) sites which show an increase in 3' processing activity with infected extracts are inherently less efficient than those which do not respond to the virus-associated 3' processing factor.

IE63 gene expression is required for the increase in 3' processing activity. Our previous studies have shown that the virus-associated 3' processing activity requires expression of IE63 (28). Here, we have examined the processing efficiencies of the six test HSV-1 poly(A) sites in the presence and absence of IE63 expression; nuclear extracts were prepared from HeLa cells infected with the IE63 null virus mutant 27-LacZ (44). The in vitro 3' processing efficiencies of test poly(A) sites were compared by using 27-LacZ-infected, wt (KOS) HSV-1-infected, and mock-infected extracts. Processing efficiencies of the UL38 and UL44 late poly(A) site precursors (Fig. 3, lanes 14 and 17) were of levels similar to those of both mock-infected and 27-LacZ-infected extracts, and there was a marked three- to fivefold increase in processing efficiencies with wt-infected extracts (Fig. 3, lanes 13 to 18). By contrast, the poly(A) sites from the UL29, UL23, IE110, and IE63 genes showed similar processing efficiencies with all three extracts (Fig. 3, lanes 1 to

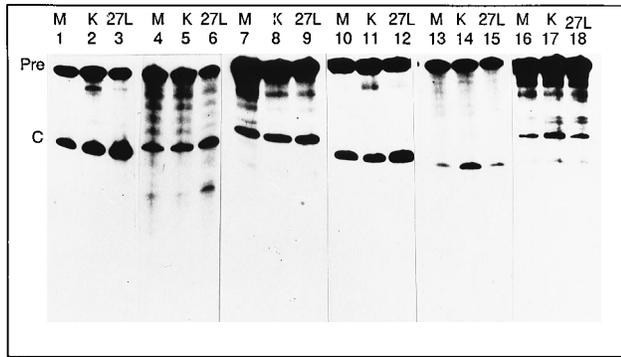


FIG. 3. The role of IE63 protein in the virally mediated increase of 3' processing shown by selected HSV-1 poly(A) sites. Nuclear extracts were prepared from mock-infected (M), HSV-1 strain KOS-infected (K), and 27-LacZ IE63 null mutant virus-infected (27L) HeLa cells. Cleavage products generated with these extracts are shown in lanes 1 to 18. Lanes 1 to 3, IE110 gene poly(A) site; lanes 4 to 6, IE63 gene poly(A) site; lanes 7 to 9, UL23 gene poly(A) site; lanes 10 to 12, UL29 gene poly(A) site; lanes 13 to 15, UL38 gene poly(A) site; lanes 16 to 18, UL44 gene poly(A) site. Pre is unprocessed precursor RNA, and C represents products generated by cleavage of the precursor RNA.

12), with 27L (lane 3) showing a slightly more intense band, within normal variation between gels. Thus, expression of IE63 is required for the observed increase in processing efficiencies of the UL38 and UL44 poly(A) sites with infected cell extracts. Processing efficiencies of the IE63, IE110, UL23, and UL29 poly(A) sites, previously shown to be unresponsive to the HSV-induced activity, are unaffected by the presence or absence of IE63.

Increased binding of proteins to HSV-1 poly(A) site mRNAs with infected nuclear extracts. The IE63-associated viral activity increased the 3' processing efficiency at selected late HSV-1 poly(A) sites. To examine whether control was related to protein binding, we determined the ability of pre-mRNAs to bind to proteins from wt-infected, mock-infected, and 27-LacZ-infected nuclear extracts.

Equal concentrations of protein from mock-infected and infected nuclear extracts were incubated with a constant amount of 32 P-labelled precursor RNA from the six test poly(A) sites and subjected to UV-irradiation cross-linking. Three main groups of proteins commonly bound to the test poly(A) site RNAs: a triplet (or doublet) of proteins (band A) of approximately 90 to 150 kDa, a protein band (B) of approximately 64 kDa, and band C of approximately 40 kDa (Fig. 4). Although there was some variability in the exact binding patterns between RNAs, the patterns were quite consistent within RNAs. These protein bands, and the binding patterns, resemble those obtained with components of the 3' processing complexes, CPSF and CstF, which have been shown to bind to other viral and cellular pre-mRNAs (8, 15, 31, 45, 52). Similar binding patterns were observed with each precursor mRNA with the exception of the UL38 poly(A) site, which consistently failed to bind protein C.

The main feature of these binding reactions was the demonstration of an overall increase in protein-RNA binding with the infected nuclear extracts compared with the mock-infected extracts. This increase in binding occurred with all three protein bands but was particularly apparent in the case of protein bands B and C (Fig. 4, lanes 2, 4, 6, 8, 10, and 12). The protein band A usually appeared as a triplet; however, this was somewhat variable. Protein bands B and C appeared as doublets; this may reflect different phosphorylated or processed forms of the same proteins. UV cross-linking experiments using nuclear

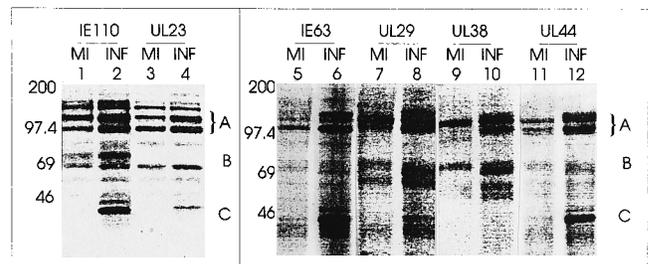


FIG. 4. RNA-protein binding of the HSV-1 test poly(A) sites with mock-infected (MI) and HSV-1 strain 17⁺-infected (INF) HeLa cell nuclear extracts, carried out by UV cross-linking analysis. The 32 P-labelled RNA-protein cross-linked complexes were separated on 10% polyacrylamide gels and visualized with a PhosphorImager. Lanes 1 and 2, the IE110 gene poly(A) site; lanes 3 and 4, UL23 gene poly(A) site; lanes 5 and 6, IE63 gene poly(A) site; lanes 7 and 8, UL29 gene poly(A) site; lanes 9 and 10, UL38 gene poly(A) site; lanes 11 and 12, UL44 gene poly(A) site. Positions of protein molecular weight (in thousands) markers are shown on the left. A, B, and C represent the major groups of proteins bound to pre-mRNAs (see text).

extracts from 27-LacZ-infected HeLa cells demonstrated a pattern of binding which resembled the mock-infected binding pattern, with all infection-induced enhancement of binding lost (Fig. 5, lanes 3 and 6). Western analysis using an anti-64-kDa protein antibody (kindly provided by C. MacDonald) has allowed us to identify a component of the upregulated band B as comigrating exactly with the 64-kDa component of CstF (Fig. 6A and B). Further, using an antipeptide antibody, we have demonstrated that IE63 protein does not bind to the pre-mRNAs in this assay (data not shown).

These results demonstrate that HSV-1 infection increases the binding of selected proteins to the mRNAs of all the HSV-1 test poly(A) sites and that IE63 protein is required for this effect.

Splicing factors are more dramatically redistributed with HSV-1 infection than are polyadenylation factors. As HSV-1 infection can cause stimulation of viral polyadenylation and an increased binding of polyadenylation factors to their substrate RNAs, and the presence of IE63 is required for this effect, we examined the intranuclear distribution of two factors involved

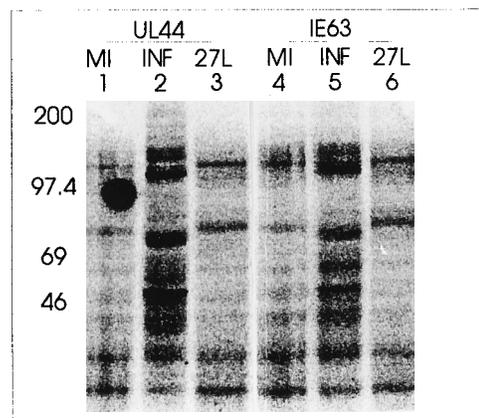


FIG. 5. RNA-protein binding of two HSV-1 test poly(A) sites with mock-infected (MI), HSV-1 strain 17⁺-infected (INF), and 27-LacZ-infected (27L) HeLa cell nuclear extracts. The 32 P-labelled RNA-protein cross-linked complexes were separated on 10% polyacrylamide gels and visualized with a PhosphorImager. Lanes 1 to 3, UL44 gene poly(A) site; lanes 4 to 6, IE63 gene poly(A) site. Positions of protein molecular weight (in thousands) markers are shown on the left.

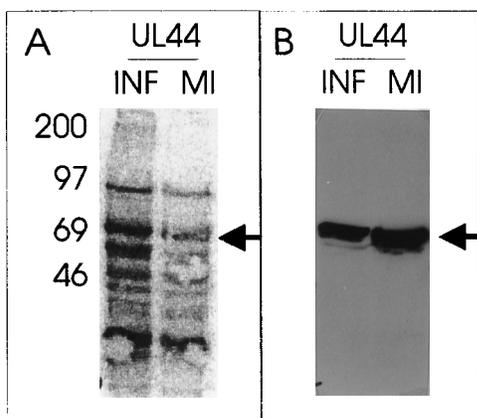


FIG. 6. Western blot analysis of proteins UV cross-linked to ^{32}P -labelled poly(A) sites. Nuclear extracts were prepared from mock-infected (MI) and HSV-1 strain 17 $^{+}$ -infected (INF) HeLa cells. UV cross-linked proteins were resolved by 10% polyacrylamide gel electrophoresis, Western blotted onto nitrocellulose membrane, and then probed with an antibody against the 64-kDa component of CstF. The poly(A) site RNA used here was from gene UL44, though identical results were obtained regardless of the poly(A) site. Positions of protein molecular weight (in thousands) markers are shown on the left. (A) All radiolabelled cross-linked proteins, as visualized with a PhosphorImager; the arrow indicates the position of the 64-kDa CstF protein. (B) Western blot with the anti-64-kDa protein monoclonal antibody with the ECL detection system.

in posttranscriptional RNA processing, namely, the B component of the U2 splicing snRNP and the 64-kDa component of the CstF polyadenylation factor, by indirect immunofluorescence.

The distribution of the 64-kDa polyadenylation factor was smooth with a slight ringing around the nucleoli in mock-infected cells (Fig. 7a); by 16 h postinfection, the polyadenylation factors had slightly clumped within the nucleus (Fig. 7b). The cell shown in Fig. 7b is quite a dramatic example of the

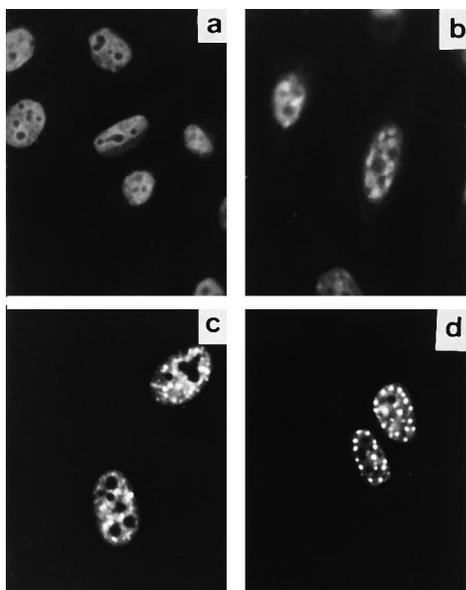


FIG. 7. Intranuclear locations of the U2 snRNP and the 64-kDa polyadenylation factor. Shown are indirect immunofluorescence photomicrographs of mock-infected HeLa cells (a and c) and HeLa cells 16 h postinfection with HSV-1 strain 17 (b and d), labelled with antibodies against the 64-kDa component of CstF (a and b) and the B' protein component of U2 snRNP (c and d).

extent of polyadenylation factor clumping; it is not unusual to see a less pronounced effect (35).

As we have demonstrated previously (35), HSV-1 infection causes the snRNPs required for RNA splicing to be redistributed away from the general diffuse pattern seen in mock-infected cells (Fig. 7c) to a highly punctate pattern (Fig. 7d). IE63 protein alone can cause this effect.

Viral protein synthesis and effects of IE63 expression. IE63 is responsible for the virus-mediated upregulation of 3' processing efficiency at certain late poly(A) sites, suggesting a role for IE63 in the switch from early to late gene expression at the posttranscriptional level. To determine which genes were affected in vivo by IE63, we have examined viral protein expression in the presence and absence of this protein.

Protein extracts were prepared from HSV-1-infected and 27-LacZ-infected Vero cells. A comparison of viral protein expression over a 24-h time course of infection was made. Western analysis demonstrated that a functional IE63 gene product influenced the expression of a number of viral proteins. We have shown that wt IE63 expression is detectable in this system from 4 h postinfection and, as a negative control, that no IE63 protein is produced by 27-LacZ virus infections (Fig. 8A). In the absence of IE63 expression, the level of IE110 protein was reduced (Fig. 8B); an initial reduction in the level of UL29 protein and a delay in UL42 expression were also observed (Fig. 8C and D, respectively). However, two early proteins, the products of US6 and US8 genes (glycoproteins D and E, respectively), showed no IE63 requirement for their expression (Fig. 8E and F). True late proteins of the UL38 and UL44 genes (glycoprotein C) and the US11 genes showed an absolute requirement for IE63 expression (Fig. 8G, H, and I). In addition, the level of UL45 protein, a late protein which is 3' coterminal with the UL44 gene, was much reduced in the absence of IE63 (Fig. 8J).

As an additional control, protein extracts were prepared from 27-LacZ-infected Vero 2.2 cells. The Vero 2.2 cell line is the complementing cell line for the 27-LacZ virus and carries the IE63 gene under the control of an HSV-1 IE promoter (44). Under these conditions, IE63 expression was delayed until 8 h postinfection (Fig. 9A) and there were a concomitant reduction in UL29 protein expression at 8 h postinfection (compare Fig. 8C and Fig. 9B) and a delay in UL42 protein expression (compare Fig. 8D and Fig. 9C).

Thus, IE63 protein is required for the expression of true late genes at the appropriate time point of the infectious cycle; implicit in this is the requirement of IE63 for viral DNA synthesis.

DISCUSSION

We have focused our attention on the roles that 3' processing of mRNA and the differential use of poly(A) sites may play in the regulation of HSV gene expression and have further characterized the virus-induced 3' processing activity identified by McLauchlan et al. (29), which is capable of increasing usage of selected HSV poly(A) sites. An in vitro assay system which measures the efficiency of mRNA cleavage, a marker of 3' processing efficiency, was used to determine the efficiency of selected HSV-1 poly(A) sites while having no effect on the poly(A) sites of two IE and two early HSV-1 genes. Expression of the IE63 protein was required for this virally mediated effect on 3' processing, and this is in accordance with previous data which demonstrated, with a range of virus mutants deficient in IE gene expression and by transient transfection studies, that this

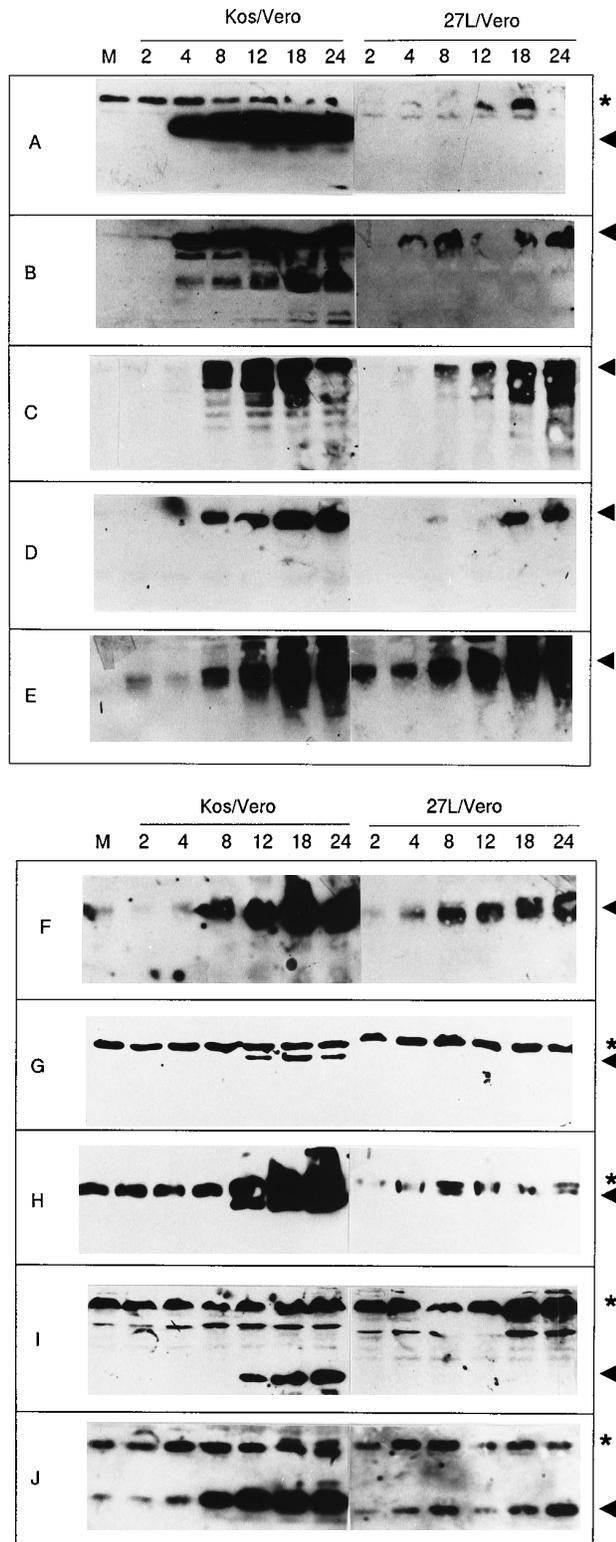


FIG. 8. Western blot analysis of HSV-1 proteins made in the presence and absence of IE63 expression. Extracts were prepared from HSV-1 strain KOS-infected Vero cells (KOS/Vero) and 27-LacZ-infected Vero cells (27L/Vero) at 2, 4, 8, 12, 18, and 24 h postinfection. Extracts were probed with the following antibodies: anti-IE63 (A), anti-IE110 (B), anti-UL29 (C), anti-UL42 (D), anti-US6 (E), anti-US8 (F), anti-UL38 (G), anti-UL44 (H), anti-US11 (I), and anti-UL45 (J). M is mock-infected Vero cell extracts. Arrowheads indicate ECL-detected antigens. Asterisks indicate that bands are due to cross-reaction with cellular proteins.

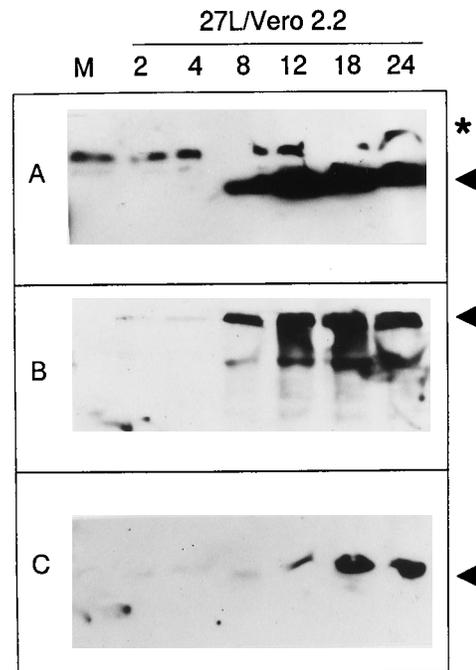


FIG. 9. Western blot analysis of HSV-1 proteins made in complementing 2-2 cells infected with the IE63 mutant virus 27-LacZ. Extracts were prepared from 27-LacZ-infected Vero 2-2 cells at 2, 4, 8, 12, 18, and 24 h postinfection. Extracts were probed with anti-IE63 antibody (A), anti-UL29 antibody (B), and anti-UL42 antibody (C). M is mock-infected Vero 2-2 cell extracts. Arrowheads indicate ECL-detected antigens. The asterisk indicates that the band represents cross-reaction with a cellular protein.

gene product was essential for upregulation of poly(A) site usage (28).

Several studies have demonstrated that the IE63 protein is involved in the regulation of HSV gene expression (11, 30, 39, 43, 44) and is specifically required for late gene expression (13, 39). The plethora of conflicting information and the apparently multifunctional nature of the IE63 protein have, however, made identification of the specific mechanism(s) of this regulation difficult. Our data indicate that IE63 may influence late gene expression via an ability to increase the 3' processing efficiency of some late HSV genes. A caveat is that the HSV-2 poly(A) site of the US10, US11, and US12 genes which did not respond to the IE63-dependent activity is utilized by three genes of the IE, early, and late temporal classes. Hence, IE63 does not act by increasing pre-mRNA 3' processing of all late genes. A striking feature of poly(A) sites which responded to the IE63-dependent activity was their inefficient basal levels of 3' processing activity. If weak poly(A) sites are a general, but not exclusive, feature of late poly(A) sites, this effect could regulate viral gene expression by reducing unscheduled late gene expression at early times. As infection proceeds, activity of the cellular proteins required for 3' processing is likely to decline and IE63 would boost processing at later times. Alternatively, the IE63-mediated activity could increase 3' processing at all virus poly(A) sites in vivo, with the effect being apparent only in vitro at the weaker poly(A) sites.

We do not rule out the possibility of effects on transcription acting to regulate expression of late viral genes. Here, efficiencies of 3' processing were determined in the absence of any influence that transcriptional control might exert. Enhancement of late viral gene expression may require additional factors which complement posttranscriptional control, such as the

additional promoter sequences which Guzowski et al. (10) found to be a feature of several late genes and which act to enhance their expression at later stages of infection.

Examples of posttranscriptional regulation of gene expression in which poly(A) site choice is influenced both by inherent poly(A) site strength (efficiency of 3' processing) and by the concentration of processing factors have been described for both mammalian and viral systems. During B-cell maturation, the switch from production of the membrane-bound (mm) to the secretory form (ms) of immunoglobulin M results at least in part from alternative poly(A) site selection (5, 9, 16, 34). Another example is the differential use of the adenovirus L1 and L3 poly(A) sites at early and late times. A proposal is that poly(A) site selection is determined by differing strengths of the L1 and L3 sites; at late times of infection when one of the RNA processing factors is present at a limiting concentration, the stronger L3 (late) site is used in preference to the weaker L1 (early) site (20).

We studied viral protein expression from an IE63-deficient mutant virus to examine the role of IE63 *in vivo*. Expression of the true late genes US11, UL38, UL44, and UL45 required the IE63 protein. These results are in agreement with the general pattern of expression observed in other studies which show a reduction in late gene expression in the absence of IE63 (36, 39, 44). The other genes examined which were affected by the absence of IE63 either are involved in DNA synthesis (UL29 and UL42) or have a known regulatory role (IE110); they did not display an absolute requirement for IE63 expression, but rather, protein levels were reduced or delayed. Uprichard and Knipe (46) demonstrated an underexpression of the UL5, UL8, UL9, UL42, and UL30 genes in ICP27 mutant virus infection. Similar reductions in the level of protein expression and a delay in production of these proteins were noted when the virus mutant was used to infect the complementing cell line 2.2 (containing an integrated copy of the IE63 gene which can be transactivated by HSV infection). The delay in IE63 expression from 4 to 8 h in these cells corresponded with a delay in UL42 expression, a reduction in UL29 expression, and a reduction in IE110 expression at 4 h postinfection. By affecting expression of these proteins, IE63 could disrupt the normal sequence of events required for efficient DNA synthesis and, subsequently, the DNA replication-dependent activation of late gene expression; also, in IE63 mutant virus-infected cells, assembly of prereplicative complexes in the nucleus is defective, because of an observed conformational change in the UL29 protein (3). This proposed role for IE63 in the efficient induction of viral DNA synthesis could explain the finding that, while IE63 is required for US11 expression, it has no effect on the 3' processing efficiency of the US11 poly(A) site.

RNA-protein cross-linking studies showed a distinctive pattern of protein binding, and sizes of the protein bands correlated well with components of the mammalian CPSF and CstF complexes required for 3' processing (8, 31, 48). The <69-kDa cross-linked protein was identified as the 64-kDa protein of the CstF complex which binds to a U-rich motif in RNA located immediately downstream of the 3' cleavage site and is essential for formation of a functional polyadenylation complex (19). Increased binding of this component could be the means by which HSV infection increases 3' processing. Whether IE63 interacts with the 64-kDa CstF component is not known, though we know that IE63 itself is not one of the cross-linked proteins, though IE63 does bind RNA weakly. There are dramatically reduced levels of proteins cross-linked to RNA with nuclear extracts prepared from cells infected with the 27-LacZ virus compared with wt-infected extracts, indicating that IE63 is required for increased binding of 3' processing factors.

In the mammalian system, assembly of the 3' processing complex and subsequent cleavage and polyadenylation of pre-mRNA require the presence of specific sequence elements in the 3' region of the RNA. Binding of the CPSF complex and of poly(A) polymerase is via direct interaction with the poly(A) signal AAUAAA (7, 48); the subsequent binding of the CstF complex, which is dependent on GU- or U-rich tracts present in the region downstream of the AAUAAA, stabilizes the CPSF interaction (19, 50). It is thought that CstF binding determines the efficiency of polyadenylation (7, 8, 45, 48). We have examined the RNA sequences of HSV-1 poly(A) sites for the presence of GU- or U-rich tracts 3' to the poly(A) signal. Of the 47 HSV-1 poly(A) site sequences examined (Table 1), 45 contained GU-rich elements, 19 of which contained one or more elements conforming to the consensus (YGUGUUY) identified by McLauchlan et al. (27), and 21 poly(A) sites contained one or more elements conforming to the core of the consensus (RGUUYRR) identified by Sadofsky and Alwine (40) or the U-rich tract identified by MacDonald et al. (19). Activity of the downstream element is dependent on its position: increasing the positioning to more than 40 nucleotides 3' of the AAUAAA abrogated its effect (2, 6, 24, 55). The majority of the elements present in HSV-1 poly(A) sites were found to lie within 20 to 35 nucleotides of the AAUAAA (Table 1). No definitive pattern either of nucleotide composition or of distance of the elements 3' of AAUAAA was discerned in the six test poly(A) sites used here or indeed among HSV-1 poly(A) sites from the same temporal class. Thus, there is no clear evidence of downstream RNA sequences which might account for the differences in processing efficiency of the HSV-1 poly(A) sites.

Moreira et al. (32) have identified a class of poly(A) site activation sequences in the form of U-rich upstream elements (USE). These are located 5' to the AAUAAA signal and confer efficient usage of poly(A) sites. Examination of all HSV-1 poly(A) sites has revealed that 70% have forms of these USE consisting of three to five U residues which are present either singly or in three to four copies; the distance of these USE varies from 3 to 98 nucleotides 5' of the AAUAAA. As the number and position of these elements are highly variable, it is difficult to draw a relationship between the type of USE and efficiency of the poly(A) sites examined here. However, the three inherently weak poly(A) sites from HSV-1 UL38 and UL44 and HSV-2 UL38 had very poor, if any, USEs. This could possibly account for their low basal levels of usage but does not account for their upregulation of usage in the presence of IE63 protein.

As well as effects on RNA 3' processing, IE63 acts to redistribute the nuclear splicing snRNPs from a widespread speckled arrangement to a punctate pattern. This results in a concomitant inhibition of splicing, which inhibits cellular gene expression while having only minimal impact on the expression of viral genes, which are for the most part unspliced (25). However, throughout infection the distribution of the polyadenylation factors such as the 64-kDa component of CstF and the 160-kDa component of CPSF remains largely unaltered. *In vitro* and *in vivo* studies have shown that splicing and polyadenylation are linked; the two processes interacting to define the 3'-terminal exon of spliced RNAs and with splicing increasing the efficiency of polyadenylation (18, 33). In HSV-infected cells, because of the action of the IE63 protein, the processes of polyadenylation and splicing are uncoupled, and the redistributed snRNPs are located away from sites of transcription and polyadenylation of virus RNA (data not shown). We have found that the majority of intronless viral mRNAs are transported to the cytoplasm, while at late times postinfection pre-

TABLE 1. GU- and U-rich tracts located 3' to HSV-1 gene poly(A) signals

Gene class	HSV-1 gene	GU/U-rich element	Distance 3' of AAUAAA ^a
IE	IE110	UUUACUUUUUGUAUGUUUU	6
		UGUGUUUGG	31
	IE175	GUUUUG	22
		GUUGUUUA	35
	IE63	GUGUUGUCCUUCUUU	33
	IE68	GUUUUGU	21
		GUGUUUGGGGUUU	82
	IE12	UUUU	4
		UUUUAU	17
		GUGUCUU	29
		UUUGUGUGUUGUU	24
	Early	UL8/9	GUGUGUG
UL23		GGGUGUUUGGGUCGUUUUGUU	11
UL27/28		GGGGUGUU	10
		UUUUUGGUUUUGUUUGUUUGGU	33
		UUUUUGUGUGUGUGGG	57
UL29		UUGUUUGUGUU	25
UL30		GUGGGUUU	39
UL39/40		UUUGGGU	18
		UUGUAUUCUUGU	29
UL42		UUGUAUUG	5
		GUGUGGUUCAUUGUGUGG	37
		GGGUUCGUGUAUUUCCUUU	73
UL49/49A		GGUGUUUGG	25
		UUUGUUU	43
UL50		GUUUUGU	8
		UGUGUGUAGUUGUUUAUGUUGG	27
Early-late	UL11/14	GUGUCGUUUUU	37
	US5/7	UAUUUUUU	8
		GUGUAUGU	26
		UUUCUUU	37
	UL1/2	UGGUU	13
		GGUGAUG	41
	UL1/3	UUUGGCUGGUUGUUGUUG	15
	UL15	GGUGUCGUCUGUU	33
		GUGUUUG	65
	UL16/17	UGGUUGUGUGGUAUGU	40
	UL18/20	UGUGUGU	30
		UUUGGGUUGGGUUUCUGUGUU	51
	UL24	GUGUGUUUUUUU	41
Early-late	UL24/25	UUCUUUUUUUUUU	37
	UL37	GUGUGUUUGUUCUGGUGUU	29
	UL41	UGGUU	41
		CUUUUUG	51
		CUUUUAU	63
	UL48	GGUUGU	31
		UUUGGUGGUGGUGGGGG	47
	UL51	GUGUUUU	7
		GUGUGUUUUUGUGUUUGUGGG	24
	US2	GUGGUCUGUUUCUCU	28
		UUUGAUUUUGGUCUUUU	79
	US3/4	GGUGGU	7
		UGGGUGUUUUUGGGGUGU	33
	US8/9	UUGGGUU	4
	UGUUGU	21	
	AUUUUUG	38	
True late	UL10	GUUUUGUGUUU	6
		UGUGGUUUUGUUUGUU	15
		UUUGCUUU	65
	UL31/32	UUUUU	6
	UL38	GUGUUUGUAACGUCCUUU	26
	UL38	UGUGUUUGGCGUGUGUCUCUG	26
	UL44/45	GUGGUGUUUUUGUUUAUUUUU	43

Continued on following page

TABLE 1—Continued

Gene class	HSV-1 gene	GU/U-rich element	Distance 3' of AAUAAA ^a
	UL46/47	<u>GUGUUCUUUUU</u> <u>UUGUGUGUUUAUUUUU</u>	32 54
Class unknown	UL4/5	UUUAUGUGUUUUUUUU	25
	UL6/7	GGUUUUGUUU UUGGUGGGUGGGUGGCUGUGU	7 56
	UL43	GGUGUGUG	27
	UL52/53	UUAUGGUGU	18
	UL55	GUGGUGUGAGUUUUGUGG	29

^a Distance (in nucleotides) of the first nucleotide of each GU/U-rich element 3' to the U of AAUAAA. Underlined sequences correspond to the core of the consensus sequence YGUGUUYY described by McLauchlan et al. (27) where Y = pyrimidine.

mRNAs from the small number of spliced genes are retained within clumps of the redistributed snRNPs (data not shown). It is possible that the effect of IE63 on 3' processing could result from its effect on the splicing components. Reorganization of the splicing components, but not of the polyadenylation factors, away from the sites of transcription may create an apparent localized increase in 3' processing factors such that pre-mRNAs are more accessible to interaction with components of the 3' processing machinery. This property would have to be maintained by the infected nuclear extracts, and it is possible that clumping of snRNPs is maintained in active 3' processing extracts. Alternatively, IE63 protein could induce a general increase in the levels of 3' processing components.

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