Alternatively spliced mRNAs predicted to yield frame-shift proteins and stable intron 1 RNAs of the herpes simplex virus 1 regulatory gene α0 accumulate in the cytoplasm of infected cells

(control of RNA longevity/regulation of splice acceptor sites)

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ABSTRACT   The infected cell protein no. 0 (ICP0), the product of the α0 gene, and an important herpes simplex virus 1 regulatory protein is encoded by three exons. We report that intron 1 forms a family of four stable nonpolyadenylylated cytoplasmic RNAs sharing a common 5' end but differing in 3' ends. The 5' and 3' ends correspond to the accepted splice donor and four splice acceptor sites within the mapped intron domain. The most distant splice acceptor site yields the mRNA encoding the 775-aa protein known as ICP0. The mRNAs resulting from the use of alternative splice acceptor sites were also present in the cytoplasm of infected cells and would be predicted to encode proteins of 152 (ICP0-B), 87 (ICP0-C), and 90 (ICP0-D) amino acids, respectively. Both the stability of the α0 mRNA and the utilization of at least one splice acceptor site was regulated by ICP22 and or $U_S1.5$ protein inasmuch as cells infected with a mutant from which these genes had been deleted accumulated smaller amounts of α0 mRNA than would be predicted from the amounts of accumulated intron RNAs. In addition, one splice acceptor site was at best underutilized. These results indicate that both the splicing pattern and longevity of α0 mRNA are regulated. These and other recent examples indicate that herpes simplex virus 1 regulates its own gene expression and that of the infected cells through control of mRNA splicing and longevity.

The herpes simplex virus 1 (HSV-1) encodes at least 84 different genes (1–4) expressed in three coordinately regulated, sequentially ordered groups designated α, β, and γ (5). Expression of α genes, the first set of genes to be expressed, does not require prior protein synthesis (5, 6). For the most part, the products of these genes regulate viral gene expression. This report concerns three α genes, α0, α22, and $U_S1.5$. The product of the α0 gene, the infected cell protein no. 0 (ICP0), is best described as a promiscuous transactivator (7–14). In the absence of α0, the virus multiplies at high multiplicities of infection but infection is sluggish and the virus is unable to reactivate efficiently from sensory ganglia harboring latent virus (8, 10, 12–14). The currently accepted coding domain signaled by the first methionine consists of three exons of 19, 222, and 534 codons, respectively (15). The mechanism by which ICP0 acts is not known. The domain of the α22 gene yields two transcripts (3, 16). The first derived by splicing of 5' sequences to an exon encoding the coding sequences, yields ICP22 (16). The second transcript, initiated within the coding domain of the α22 gene contains a truncated ORF that on translation yields a protein designated $U_S1.5$ (3). Recombinant viruses lacking the 3' domain of the α22 gene and the coding sequence of the $U_S1.5$ gene ($α22^-/U_S1.5^-$) grow in continuous cell lines but exhibit reduced capacity to replicate in primary human fibroblasts and in cells of rodent derivation (17, 18). At

the molecular level, $α22^-/U_S1.5^-$ virus-infected cells exhibit a decrease in the expression of α0 and of a subset of late (γ) genes (3, 19). ICP22. and that α22 and $U_S1.5$ proteins are phosphorylated by both HSV-1 kinases encoded by U_L13 and U_S3, respectively (ref. 20; W. Ogle, K.L.C., and B.R., unpublished studies). The posttranslational processing mediated by U_L13 is particularly important inasmuch as the phenotype of U_L13^--infected cells is similar to that of the $α22^-/U_S1.5^-$ infected cells (19).

The studies described in this report stemmed from the evidence that α22 and $U_S1.5$ genes encode functions that regulate the expression of α0 (3, 19). Although α0 is known as a highly important protein for efficient replication of HSV-1 (8, 10, 12–14), the available data on the structure and stability of its mRNAs are scarce (15, 21). In the course of these studies we found and report the following. (*i*) Intron 1 accumulated in the cytoplasm in a stable, nonpolyadenylylated form. The intron RNA appeared to be more stable than the authentic α0 mRNA. (*ii*) Intron 1 RNAs originated from one splice donor site and as many as four splice acceptor sites. (*iii*) Utilization of all four splice acceptor sites would yield α0 mRNAs that, upon translation, would yield proteins containing 775 [previously recognized as ICP0, renamed (ICP0-A), 152 (ICP0-B), 87 (ICP0-C), and 90 (OCP0-D)] amino acids, respectively. We report the presence of at least one RNA corresponding in size to alternatively spliced α0 RNA in the cytoplasm of infected cells.

MATERIALS AND METHODS

Cells and Viruses. The source and procedures for cultivation of Vero and rabbit skin cell lines and the properties of the prototype HSV-1 strain HSV-1(F) were described elsewhere (3, 4, 17). The genetically engineered recombinant virus HSV-1(Δ305) lacks the 501-bp SacI–BglII fragment from the BamHI Q fragment encoding the viral thymidine kinase (*tk*) gene, whereas R325 virus derived from HSV-1(Δ305, in addition, lacks the 3' half of the α22 gene (17).

Construction of Plasmids. Plasmid pRB3710 contains the SacI–PstI restriction endonuclease fragment of one copy of the α0 gene of HSV-1(F) in the vector pBR322 (19). pRB4919 contains the KpnI–SacI restriction endonuclease fragment of pRB3710 inserted into KpnI–SacI digested pGEM3ZF+ vector such that the T7 promoter drives transcription of the strand encoding the α0 gene. pRB4998 contains sequences specific for intron 1 of α0 generated by PCR amplification of pRB3910 using the primers 5'-CCCGCGTCAATCAGCACCCACGAGTTCCCC-3' and 5'-CTGGGGCGCCATATTGGGGGGCGCCATATT-3'. Plasmid pRB3910 contains U_S11 specific sequences (22).

Isolation of RNA. Rabbit skin or Vero cells were exposed to 10 plaque-forming units of the indicated virus per cell and harvested 12 hr after infection. Cytoplasmic RNA was extracted as previously described (23) and digested with amplification grade DNase (GIBCO/BRL) according to the manufacturer's instructions. For purification of poly(A)$^+$ RNA, total cellular RNA was extracted as described (24). RNA extracted from a 150 cm^2 flask was applied to a single magnabead poly(A)$^+$ selection column (Promega) and poly(A)$^+$ RNA was selected according to the manufacturer's instructions.

Analysis of denatured, electrophoretically separated RNA. Twenty micrograms of either cytoplasmic RNA or poly(A)$^+$ selected RNA from a 150-cm^2 flask was loaded per lane and separated in a 1.5% agarose gel containing 6% formaldehyde. The RNA was then transferred to Zeta-probe membrane (Bio-Rad) in 10× standard saline citrate (1.5 M NaCl plus 0.15 M trisodium citrate). The blots were air-dried and baked at 80°C in a desiccating oven. Riboprobes were generated using the plasmid templates indicated below and SP6 polymerase with the Gemini riboprobe system (Promega) according to the manufacturer's directions. Nick-translated probes were generated with the indicated plasmids using a kit purchase from Promega. Hybridizations were done at 68°C with nick-translated DNA probes or at 85°C with riboprobes overnight in hybridization solution (1 mM EDTA/5 M NaH$_2$PO$_4$, pH 7.2/7% SDS).

S1 Analyses. Cytoplasmic RNA (20 μg) was hybridized with either the 5′ or 3′ α0 intron 1 specific probe in hybridization solution (80% formamide/0.4 M NaCl/1 mM EDTA/40 mM Na$_2$/Pipes, pH 6.4). The 5′-specific probe was generated by PCR amplification of pRB3910 using the oligonucleotides 5′-TGTCACGGGGTTCCACGCCCCCTAACATGG-3′ and 5′-CAGAAGCCCCGCCTACGTTGCGACCCCCAG-3′. The fragment was purified with the wizard PCR purification kit (Promega) and 5′ end-labeled with [^{32}P]ATP (Amersham) and polynucleotide kinase (United States Biochemical). The 3′ probe was generated by gel purification of the 988-bp XmaI endonuclease fragment from pRB4919. The cleavage left a 3′ recessed end whose next templated nucleotide would be a cytosine. The fragment was 3′ end-labeled with [α^{32}P]dCTP (Amersham) and the Klenow fragment (Boehringer Mannheim). The hybridization was carried out overnight at 58°C using the conditions previously described (3). The products were separated on a 6% polyacrylamide/7 M urea gel. Control reactions contained no input RNA.

RESULTS

The 5′ and 3′ Termini of a Cytoplasmic RNA Correspond to the Donor and Acceptor Sites of Intron 1 of the α0 Gene. Preliminary experiments showed that a cytoplasmic RNA hybridized to a probe containing sequences mapped in the intron 1 of the α0 gene (data not shown). The purpose of the experiments detailed below were to map the 5′ and 3′ termini of this RNA to ascertain whether it in fact contained the intron 1 of the α0 gene. If the new transcript were stable α0 intron 1, it would be expected that its 5′ and 3′ ends would correspond to the spliced donor and acceptor sites of that intron. In this series of experiments the probes described in *Materials and Methods* and in Fig. 1 were hybridized to cytoplasmic RNA and S1 analyses were done as described in *Materials and Methods*. The results were as follows.

(*i*) S1 nuclease protection analyses mapped the 5′ end of the new transcript precisely to the splice donor signal of α0 exon 1. Band F in Fig. 2, lanes 7–10, showed a protected species migrating at ≈210 bp, which is the expected size of the protected species if the new transcript were a stable RNA containing the α0 intron 1 sequences. The transcripts of both
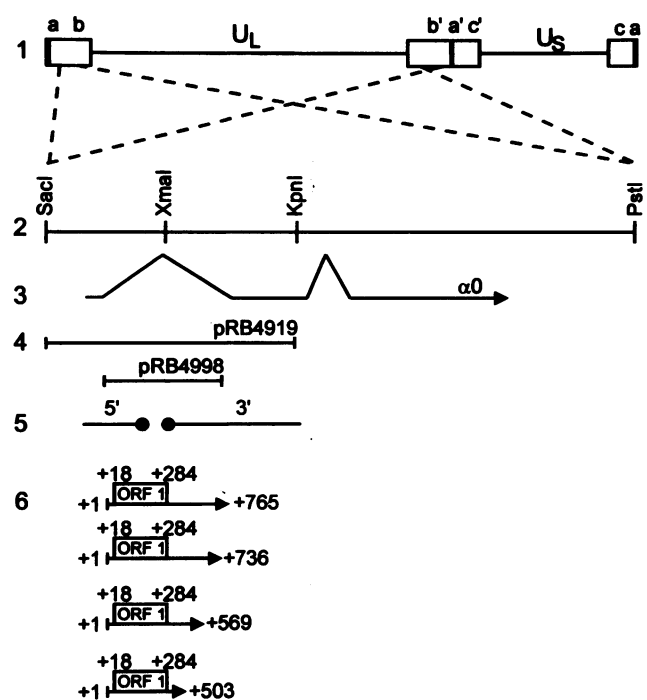
FIG. 1. Schematic diagram of the domain of the α0 genes. Line 1 is a linear representation of the HSV-1 genome. The unique sequences are represented as the unique long (U$_L$) and unique short (U$_S$) regions. The terminal repeats flanking the unique sequences are shown as open rectangles with their designation letters above. Line 2 is an enlargement of the α0 region found in each of the *b* sequences and contained in the plasmid pRB3910. Restriction endonuclease sites relevant to these studies are indicated. Line 3 shows the previously reported α0 transcript. Line 4 represents the two templates used to generate riboprobes in these studies. pRB4919 contains sequences corresponding to the SacI–KpnI restriction endonuclease sites of pRB3910. pRB4998 contains sequences specific to intron 1 of α0 generated by polymerase chain reaction. Line 5 indicates the S1 protection assay probes used in these studies. The 5′ probe was generated by PCR and the labeled end of the 3′ probe was generated by XmaI digestion of pRB4919. Line 6 depicts the four α0 intron 1 species indicated by S1 protection analyses. All four of the species have a common 5′ end labeled here as +1. Each of the four species have a different 3′ end labeled with the ending nucleotide relative to +1. All four of the species contain a common ORF designated ORF1 that resides at nucleotides +18 to +284 relative to the +1 site shared by all of these transcripts.

wild-type and R325 viruses yielded identical 5′ ends in both Vero and rabbit skin cell lines (Fig. 2, lanes 7–10).

(*ii*) The larger protected species marked band E that had the size of the full-length species resulted from hybridization to the unspliced α0 transcript and was not an artifact due to the probe hybridizing to itself, since a reaction with probe alone and no input RNA did not show a protected species (Fig. 2, lane 11).

(*iii*) The 3′ end of the new transcript as determined by S1 nuclease protection analyses mapped to a family of splice acceptor signals at the 5′ end of exon 2 of α0. The largest protected species, which formed a doublet at ≈400 bp, showed 3′ ends that mapped to the reported splice acceptor site of exon 2 and a site 19 bases upstream of that site (Fig. 2, bands A and B, lanes 2–5). In addition, a protected fragment of ≈195 bp was present in extracts from wild-type virus-infected cells of both cell lines, while a protected fragment of ≈165 bp was present in all extracts (Fig. 2, bands C and D, respectively, lanes 2–5). None of these protected species were artifacts due to the probe hybridizing to itself since they were not seen in reaction mixtures containing the probe alone (Fig. 2, lane 6).

We conclude that an RNA corresponding to intron 1 of HSV-1 α0 gene is stable and transported to the cytoplasm. This

FIG. 2. Autoradiographic images of ^{32}P-labeled probes protected from S1 degradation of cytoplasmic RNA to determine the 5' and 3' ends of the stable intron 1 RNA accumulating in the cytoplasm. All reactions except those of the probe alone contained 20 μg of input RNA. Lane 1 is MspI-digested pBR322 DNA that serves as a marker with the sizes of its fragments indicated at the le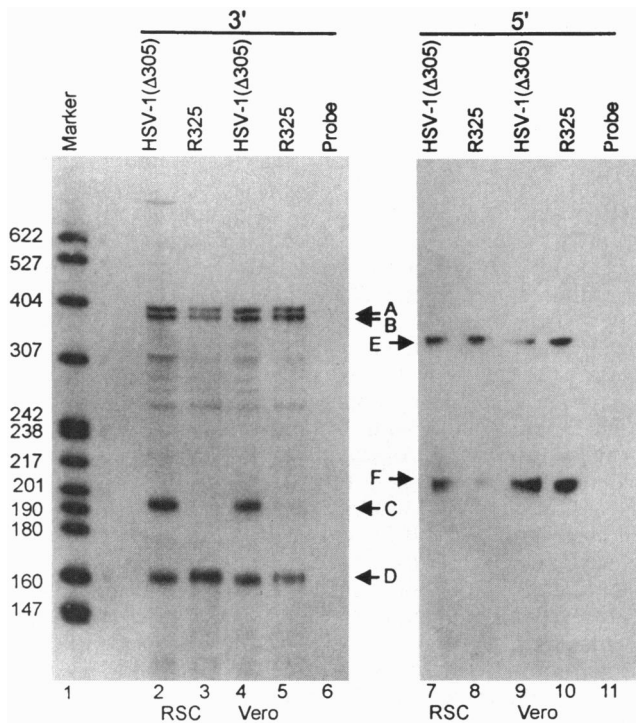ft. Reactions to map the 3' end of the transcript are in lanes 2–6. Reactions to map the 5' end of the transcript are in lanes 7–11. Lanes 2 and 7 contain RNA extracted from rabbit skin cells infected with HSV-1(Δ305). Lanes 3 and 8 contain RNA extracted from rabbit skin cells infected with R325. Lanes 4 and 9 contain RNA extracted from Vero cells infected with HSV-1(Δ305). Lanes 5 and 10 contain RNA extracted from Vero cells infected with R325. Lanes 6 and 11 contain no input RNA. Letters A–F indicate protected species.

RNA, designated α0 intron 1 RNA, arises from a single splice donor and several splice acceptor sites, and therefore the results predict that the α0 mRNA is heterogeneous with respect to the presence of sequences at the 3' end of intron 1. Inasmuch as band C was absent or grossly diminished in rabbit skin cells and in Vero cells infected with α22$^-$/U$_S$1.5$^-$ mutant R325, we conclude that α22 and or U$_S$1.5 control the utilization of the splice acceptor site C.

The α0 Intron 1 RNAs Have Altered Mobility in Extracts of Rodent Cell Lines Infected with the Recombinant Virus R325. The objective of these studies were to measure the relative amounts and electrophoretic mobility of α0 and α0 intron 1 RNAs in cell lines infected with HSV-1(F)Δ305 and R325. Two series of experiments were done. In the first series electrophoretically separated cytoplasmic RNA extracted 12 hr after infection of Vero or rabbit skin cells was hybridized to a strand-specific riboprobe antisense to the first two exons and first intron of α0 (Fig. 1, line 4). The results (Fig. 3A) were as follows.

(i) The probe hybridized with a 2.5-kb transcript present among RNAs extracted from all infected cells and representing the mature, fully processed α0 mRNA. This transcript was present in markedly higher amounts in cells infected with the parent HSV-1(F)Δ305 than in cells infected with the α22$^-$/U$_S$1.5$^-$ R325 mutant. For reasons as yet to be determined, this transcript has slightly faster mobility in infected rabbit skin cell extracts than in Vero extracts.

(ii) The probe also detected among RNAs extracted from both cell lines four RNA species estimated to be ≈800 bases in size (Fig. 3A, lanes 1 and 3). The same probe reacted with a faster migrating species in extracts of rabbit skin cells infected with the R325 virus (Fig. 3A, lane 2) than in extracts of Vero cells infected with the same virus.

In the second series of experiments the same RNAs were hybridized with a strand-specific riboprobe consisting of sequences entirely antisense to intron 1 of the α0 gene. The results were as follows.

(i) The intron specific riboprobe reacted strongly with broad bands corresponding to the α0 intron 1 RNA, and in small amounts, with the 2.5-kb spliced α0 transcript in extracts of HSV-1(Δ305)-infected cells (Fig. 3B, lanes 1 and 3). The α0 intron 1 RNAs were not detected with a strand-specific riboprobe antisense to exon 1 of α0 (data not shown).

(ii) The predominant species of α0 intron 1 RNAs detected in rabbit skin cells infected with the R325 virus (Fig. 2B, lane 2) migrated faster than those derived from HSV-1(F)Δ305 infected cells (Fig. 2B, lane 1).

We conclude from this series of experiments the following:

(i) The amounts of 2.5-kb mature α0 RNA in cells infected with R325 mutant are significantly lower than those present in cells infected with the HSV-1(Δ305). In contrast, we detected approximately equal amounts of cytoplasmic RNAs corresponding in size to the α0 intron 1 RNAs. These results suggest that the α0 mRNA is less stable than the α0 intron 1 RNAs.

(ii) The intron specific riboprobe reacted with a family of four cytoplasmic RNAs of approximately the size expected of the α0 intron 1 RNAs, or smaller. The predominant species of intron 1 RNA in rabbit skin cells infected with HSV-1(Δ305) was different from that present in cells infected with R325 mutant.

(iii) The hybridization of the intron 1 riboprobe with the 2.5-kb species suggests that RNAs derived by alternate splicing of intron 1, as predicted from experiments shown in Fig. 1, do accumulate in the cytoplasm. The experiments shown here do

not allow precise measurement of the amounts of alternatively spliced α0 RNAs accumulating in the cytoplasm.

The Altered Mobility of α0 Intron 1 Transcripts in Extracts of Rodent Cells Infected with R325 Is Not Due to Reduced Expression of U_S11. Earlier studies have shown that a subset of γ genes exemplified by U_S11 is underexpressed in cells infected with the α22⁻/U_S1.5⁻ R325 mutant. In addition, it has been shown that U_S11 acts as an antiterminator of transcription of at least one gene (25). To determine whether the change in electrophoretic mobility of the new transcript correlated with the expression of the U_S11 gene rather than deletion of the α22 and U_S1.5 genes, cytoplasmic RNA from rabbit skin cells or Vero cells extracted 12 hr after infection with either the parent virus HSV-1(Δ305) or the R325 mutant were electrophoretically separated and hybridized to a nick-translated DNA probe specific to U_S11. Fig. 2C shows that while U_S11 levels are decreased in extracts of R325-infected rabbit skin cells, U_S11 levels are more drastically decreased in extracts of Vero cells infected with the R325 virus. Since the change in mobility of the new transcript is either minimal or not seen after infection of Vero cells infected with R325, yet U_S11 expression is more drastically reduced in the same extracts, the change in migration of the new transcript is not an effect of reduction of U_S11 gene expression.

α0 Intron 1 Transcripts Are Not Polyadenylylated. The accumulation of α0 intron 1 mRNAs in the cytoplasm was unexpected and raised the question of whether they are polyadenylylated. To answer this question, total RNA from a 150 cm² flask of rabbit skin cells infected with either the wild-type virus HSV-1(Δ305) or R325 selected for the presence of poly(A)⁺ was electrophoretically separated on a denaturing agarose gel and hybridized to a riboprobe generated from the template pRB4919 containing sequences of exon 1 through exon 2 of α0 (Fig. 1, line 4). Fig. 4 shows that the probe detected the 2.5-kb mature spliced α0 transcript in poly(A)⁺ selected extracts of HSV-1(Δ305) infected cells, but failed to detect the α0 intron 1 transcripts. In addition, the results verified the absence or low abundance of mature α0 mRNA in rabbit skin cells infected with α22⁻/U_S1.5⁻ virus. Therefore, the α0 intron 1 RNAs accumulating in the cytoplasm are not polyadenylylated.
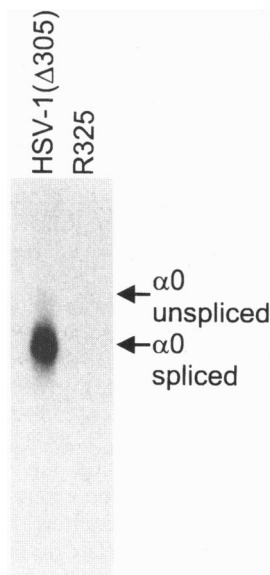
FIG. 4. Autoradiographic image of ³²P-labeled riboprobe generated from the template pRB4919 hybridized to electrophoretically separated poly(A)⁺ RNA from rabbit skin cells infected with R325 or the parent, HSV-1Δ(305) virus. Each lane was loaded with poly(A)⁺ RNA extracted from one 150-cm² flask. Lanes are labeled according to the virus that infected the cells.

DISCUSSION

It is generally accepted that the coding domain of the α0 gene consists of 775 codons distributed in three exons containing 19, 222, and 534 codons (15). With the exception one report of a truncated form of the α0 gene product accumulating in some cell lines and arising from a splicing failure in intron 2 (26), it has been assumed that the gene encodes a single protein product although the basis of this assumption, in retrospect, is not readily apparent. The salient features of this report are as follows: (*i*) The intron 1 of the α0 gene is stable, nonpolyadenylylated and accumulates in the cytoplasm. The intron appears to be more stable than the α0 mRNA—its by product. (*ii*) The electrophoretic mobility of α0 intron 1 derived from rabbit skin cells infected with the α22⁻/U_S1.5⁻ virus in denaturing gels, and by implication, its size, is slower than would be predicted from the 5′ and 3′ mapping of the RNA. (*iii*) The intron consists of several populations with an identical 5′ end but with different 3′ ends. (*iv*) Only the largest intron derived from the most distant splice acceptor site would yield the protein known as ICP0 and which, for the sake of clarity, we have renamed ICP0-A. The implication of this conclusion is that subpopulations of α0 mRNA arising by splicing through splice acceptor sites more proximal to the splice donor site must contain sequences missing from the largest intron. We present evidence that these RNAs do indeed exist and accumulate in the cytoplasm of infected cells. Relevant to these observations are the following.

(*i*) The α0 intron 1 exists as a family of four transcripts. The function of these transcripts is not known, and, because very few HSV-1 genes yield spliced transcripts, there are few precedents on which to base any hypotheses for the accumulation of intron 1 in the cytoplasm of infected cells. The only other HSV-1 intron shown to accumulate in the nucleus rather than in the cytoplasm are the latency-associated transcripts (LATs; ref. 27). These RNAs are credited with a role in the establishment and reactivation from latency (27–29) and with a non-linear structure (30). The intron 1 of α0 bears some resemblance to LATs in several respects. Like LATs, they are not polyadenylylated (29). Both LATs and intron 1 have a similar dinucleotide composition that is different from that of the rest of the viral genome (31). Like the LATs, intron 1 contains a small ORF (Fig. 1, line 6) predicted, in this instance, to encode a protein of 88 aa (32). The predicted amino acid sequence bears no significant homology to a known protein. At the nucleotide level, the intron shows a relatively low level homology to 28S ribosomal RNA.

Although S1 analyses show the same 5′ and 3′ ends irrespective of the infected cell from which they are derived, the α0 intron 1 RNA accumulating in rabbit skin cells infected with α22⁻/U_S1.5⁻ (R325) virus is significantly smaller than the corresponding RNA derived from wild-type virus-infected cells. The change in size of α0 intron 1 has yet to be explained, but one hypothesis is that it is due to a removal of sequences in the middle of the intron—a splice within a splice. The LATs again are a precedent for this type of transcriptional processing (28). In productively infected cells the predominant LAT is a 2-kb transcript. In sensory ganglia harboring latent virus there is an additional transcript with identical 5′ and 3′ termini but only 1.5 kb in size. It is thought that the internal 0.5-kb sequence is removed by a secondary splicing event.

(*ii*) Our studies show that the α0 mRNA must consist of several species differing only slightly in overall size dictated by the location of the acceptor sequence of intron 1. However, these mRNAs are predicted to express proteins vastly different in size than the 775 codon mRNA encoding ICP0-A. Thus ICP0-B, ICP0-C and ICP0-D would be predicted to contain 152, 87, and 90 aa respectively (Fig. 5B). We may only speculate on the function of these proteins in the infected cells. It is noteworthy however, that the forms described here differ from

A

↓ Transcription Initiation (+1)
GGTCGCATTTGCACCTCGGCACTCGGAGCGAGACGCAGCCAGGCAGACTCGGGCCGCCCCCTCTCCGCATCACCACA
GAAGCCCCGCCTACGTTGCGACCCCCAGGGACCCTCCGTCCGCGACCCTCCAGCCGCATACGACCCCCATGGGCCCCGCC
↓ Donor 1 (+205)
CCGGAGCGAGTACCCGCCGGCCTGAGGGCCGCCCCCAGCGCGAGGTGAGGGCCGGGCGCCATGTCTGGGGCGCCATATT
GGGGGGCGCCATATTGGGGGGCGCCCATGTTGGGGGACCCCCGACCCTTACACTGGAACCGGCCGCCATGTTGGGGGACCC
CCACTCATACACGGGAGCCGGGCGCCATGTTGGGGCGCCATGTTAGGGGGCGTGGAACCCCGTGACACTATATATACAGG
GACCGGGGGCGCCATGTTAGGGGGTGCGGAACCCCCTGACCCTATATATACAGGGACCGGGGTCGCCCTGTTGGGGGTCG
CCATGTGACCCCCTGACTTTATATATACAGACCCCCAACACATACACATGGCCCCTTTGACTCAGACGCAGGGCCCGGGG
TCGCCGTGGGACCCCCTGACTCATACACAGAGACACGCCCCCACAACAAACACACAAGGACCGGGGTCGCCGTGTTGGGG
Acceptor 1D (+706) ↓
GCGTGGTCCCCACTGACTCATACGCAGGCCCCCCTTACTCACACGCATCTAGGGGGGTGGGGAGGAGCCGCCCGCCATAT
↓ Acceptor 1C (+764)
TTGGGGGACGCCGTGGGACCCCCGACTCCGGTGCGTCTGGAGGGCGGGAGAAGAGGGAAGAAGAGGGGTCGGGATCCAAA
GGACGGACCCAGACCACCTTTGGTTGCAGACCCCTTTCTCCCCCCTCTTCCGAGGCCAGCAGGGGGGCAGGACTTTGTGA
↓ Acceptor 1B (+942)
GGCGGGGGGGGGAGAGGGGGAACTCGTGGGTGCTGATTGACGCGGGAAATCCCCCCCCATTCTTACCCGCCCCCCTTTTT
↓ Acceptor 1A (+971)
TCCCCTTAGCCCGCCCCGGATGTCTGGGTGTTTCCCTGCGACCGAGACCTGCCGGACAGCAGCGACTCTGAGGCGGAGAC
CGAAGTGGGGGGGCGGGGGGACGCCGACCACCATGACGACGACTCTGCCCTCCGAGGCGGACAGCACGGACAGGGAACTGT
TCGAGACGGGGCTGCTGGGGCCGCAGGGCGTGGATGGGGGGGCGGTCTCGGGGGGGAGCCCCCCCCGCGAGGAAGACCCC
GGCAGTTGCGGGGGCGCCCCCCCTCGAGAGGACGGGGGAGCGACGAGGGCGACGTGTGCGCCGTGTGCACGGATGAGAT
CGCGCCCCACCTGCCGTGCGACACCTTCCCGTGCATGCACCGCTTCTGCATCCCGTGCATGAAAACCTGGATGCAATTGC
GCAACACCTGCCCGCTGTGCAACGCCAAGCTGGTGTACCTGATAGTGGGCGTGACGCCCAGCGGGTCGTTCAGCACCATC
CCGATCGTGAACGACCCCCAGACCCGCATGGAGGCCGAGGAGGCCGTCAGGGCGGGCACGGCCGTGGACTTTATCTGGAC
GGGCAATCAGCGGTTCGCCCCGCGGTACCTGACCCTGGGGGGGCACACGGTGAGGGCCCTGTCGCCCACCCACCCGGAGC
CCACCACGGACGAGGATGA...

B

ICP0-B:
MetGluProArgProGlyAlaSerThrArgArgProGluGlyArgProGlnArgGluSerTyrProProProPhePhePro
LeuSerProProArgMetSerGlyCysPheProAlaThrGluThrCysArgThrAlaAlaThrLeuArgArgArgArgLys
TrpGlyGlyGlyGlyThrProThrThrMetThrThrThrProProProArgArgThrAlaArgThrArgAsnCysSerArg
ArgGlyCysTrpGlyArgArgAlaTrpMetGlyGlyArgSerArgGlyGlyAlaProProAlaArgLysThrProAlaVal
AlaGlyAlaProProLeuGluArgThrGlyGlyAlaThrArgAlaThrCysAlaProCysAlaArgMetArgSerArgPro
ThrCysAlaAlaThrProSerArgAlaCysThrAlaSerAlaSerArgAla

ICP0-C:
MetGluProArgProGlyAlaSerThrArgArgProGluGlyArgProGlnArgGluGlyGlyArgArgGlyLysLysArgGly
ArgAspProLysAspGlyProArgProProLeuValAlaAspProPheLeuProProLeuProArgProAlaGlyGlyGlnAsp
PheValArgArgGlyGlyGluArgGlyAsnSerTrpValLeuIleAspAlaGlyAsnProProProPheLeuProAlaProLeu
PheSerPro

ICP0-D:
MetGluProArgProGlyAlaSerThrArgArgProGluGlyArgProGlnArgGluProProAlaIlePheGlyGlyArgArg
GlyThrProAspSerGlyAlaSerGlyGlyArgGluLysArgLysLysLysGlySerGlySerLysGlyArgThrGlnThrThrPhe
GlyCysArgProLeuSerProProSerSerGluAlaSerArgGlyAlaGlyLeuCysGluAlaGlyGlyGlyGluGlyGluLeuVal
GlyAlaAsp

FIG. 5. Sequence of the α0 RNA and alternatively spliced products determined in these studies. (*A*) The nucleotide sequence of the 5' end of the α0 gene. Nucleotide (n) +1 corresponds to the first nucleotide of the α0 transcript. The positions of the splice donor site at n +205 and alternate splice acceptor sites n +706 (*D*), +764 (*C*), +942 (*B*), and +971 (*A*) shown were determined from S1 analyses. The dashed line identifies a sequence identical to the well conserved prokaryotic branch site. (*B*) The amino acid sequence predicted for ICP0-B, ICP0-C, and ICP0-D. The predicted amino acid sequence of ICP0-A has been published elsewhere (15). The products of the alternatively spliced RNAs share the first 19 aa.

the one described by Everett *et al.* (26) and which arises by failure of the splicing of intron 2. In that instance, a stop codon in intron 2 produced a truncated protein.

(*iii*) A key conclusion of this report is that α22 and or U$_S$1.5 regulate both the utilization of splice acceptor sites and the longevity of the α0 RNA. Control of splice acceptor site utilization emerges from the observation that site C is underused in both cell lines infected with the α22⁻/U$_S$1.5⁻ (R325) mutant virus. Control of longevity of α0 mRNA is apparent in both cell lines infected with the α22⁻/U$_S$1.5⁻ (R325) mutant virus. In principle, if both intron 1 and α0 mRNA were equally stable, we would predict that they would accumulate in equimolar amounts. In these cells the α0 mRNA is barely detectable whereas the amounts of intron 1 RNA are similar to those present in cells infected with the parent virus. Parenthetically, this laboratory reported elsewhere that ICP0 accumulates in decreased amounts in cells infected with α22⁻/U$_S$1.5⁻ (R325) mutant (3, 19). The relative paucity of α0 mRNA helps explain the apparent decrease in the amounts of ICP0. This discrepancy is particularly significant in infected Vero cells inasmuch as in these cells the α22⁻/U$_S$1.5⁻ mutant replicates to levels similar to those of wild-type virus whereas in rodent cells the yield is greatly decreased (18).

Furthermore, the results indicate that the decreased capacity of the mutant to replicate in rodent cells cannot be ascribed solely to the decreased amounts of ICP0.

The striking feature of the results presented in this report is the apparent reliance of HSV on the regulation of splicing and longevity of its RNA for the regulation of its own gene expression. The regulation of RNA longevity is apparent not only from the data presented here but also from the existence of a gene product that promotes degradation of both cellular and viral RNAs to enable an effective shift from early to late protein synthesis (33). With respect to control of splicing of RNAs, extensive experimental data from several laboratories has conclusively demonstrated that the product of the α27 gene inhibits splicing of both cellular and viral transcripts (34, 35). In addition, this laboratory reported elsewhere that the ORF P is repressed during productive infection by the major regulatory protein, ICP4 (4). Derepression of the gene by mutagenesis of the ICP4 binding site at the transcription initiation site of ORF P leads to expression of the protein (36). However, this protein is also rapidly posttranslationally processed in some cell lines but not in others. In the cell lines in which the processing is retarded, the synthesis of this protein leads to a decrease in the synthesis of ICP22 and ICP0 but not that of other α proteins (37). Although the mechanism of inhibition is not known, recent studies show that ORF P protein localizes in spliceosomes and binds a protein component of the SF2/ASF alternate splicing factor (37). The studies presented here reinforce the apparent modus operandi of the virus to control both splicing and longevity of its transcripts.

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