Alternatively spliced mRNAs predicted to yield frame-shift proteins and stable intron 1 RNAs of the herpes simplex virus 1 regulatory gene \( \alpha 0 \) accumulate in the cytoplasm of infected cells

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

KARA L. CARTER AND BERNARD ROIZMAN*

The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 East 58th Street, Chicago, IL 60637

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ABSTRACT The infected cell protein no. 0 (ICP0), the product of the \( \alpha 0 \) 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 nonpolyadenylated 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 \( \alpha 0 \) mRNA and the utilization of at least one splice acceptor site was regulated by ICP22 and of Us1.5 protein inasmuch as cells infected with a mutant from which these genes had been deleted accumulated smaller amounts of \( \alpha 0 \) 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 \( \alpha 0 \) 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 \( \alpha \), \( \beta \), and \( \gamma \) (5). Expression of \( \alpha \) 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 \( \alpha \) genes, \( \alpha 0 \), \( \alpha 22 \), and Us1.5. The product of the \( \alpha 0 \) gene, the infected cell protein no. 0 (ICP0), is best described as a promiscuous transactivator (7–14). In the absence of \( \alpha 0 \), the virus replicates 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 \( \alpha 22 \) 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 \( \alpha 22 \) gene contains a truncated ORF that on translation yields a protein designated Us1.5 (3). Recombinant viruses lacking the 3' domain of the \( \alpha 22 \) gene and the coding sequence of the Us1.5 gene (\( \alpha 22^-/Us1.5^- \)) 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, \( \alpha 22^-/Us1.5^- \) virus-infected cells exhibit a decrease in the expression of \( \alpha 0 \) and of a subset of late (\( \gamma \)) genes (3, 19). ICP22 and that \( \alpha 22 \) and Us1.5 proteins are phosphorylated by both HSV-1 kinases encoded by U13 and Us3, respectively (ref. 20; W. Ogle, K.L.C., and B.R., unpublished studies). The posttranslational processing mediated by Us1.13 is particularly important inasmuch as the phenotype of Us1.13^-infected cells is similar to that of the \( \alpha 22^-/Us1.5^- \) infected cells (19).

The studies described in this report stemmed from the evidence that \( \alpha 22 \) and Us1.5 genes encode functions that regulate the expression of \( \alpha 0 \) (3, 19). Although \( \alpha 0 \) 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, nonpolyadenylated form. The intron RNA appeared to be more stable than the authentic \( \alpha 0 \) 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 \( \alpha 0 \) mRNAs that, upon translation, would yield proteins containing 775 (previously recognized as ICP0, renamed (ICP0-A), 152 (ICP0-B), 87 (ICP0-C), and 90 (ICP0-D)) amino acids, respectively. We report the presence of at least one RNA corresponding in size to alternatively spliced \( \alpha 0 \) 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(\( \Delta 305 \)) lacks the 501-bp SacI–BglII fragment from the BamHI Q fragment encoding the viral thymidine kinase (\( \text{k} \)) gene, whereas R325 virus derived from HSV-1(\( \Delta 305 \), in addition, lacks the 3' half of the \( \alpha 22 \) gene (17).

Construction of Plasmids. Plasmid pRB3710 contains the SacI–PstI restriction endonuclease fragment of one copy of the \( \alpha 0 \) gene of HSV-1(F) in the vector pBR322 (19). pBR491 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 \( \alpha 0 \) gene. pRB4998 contains sequences specific for intron 1 of \( \alpha 0 \) generated by PCR amplification of pRB3910 using the primers 5'-CCCGCGTCGACTGACCCACCCAGGTTCGGCC-3' and 5'-CTGGGGCGCCATATTGGGGGGCCCATATT-3'. Plasmid pRB3910 contains Us1.11 specific sequences (22).

Abbreviations: HSV-1, herpes simplex virus 1; LAT, latency-associated transcripts; ICP0, infected cell protein no. 0.

*To whom reprint requests should be addressed.
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 cm² flask was applied to a single mannegabul 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-cm² 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 NaH₂PO₄, pH 7.2/7% SDS).

S1 Analyses. Cytoplasmic RNA (20 µg) was hybridized with either the 5′ or 3′ aO intron 1 specific probe in hybridization solution (80% formamide/0.4 M NaCl/1 mM EDTA/40 mM Na/Pipes, pH 6.4). The 5′-specific probe was generated by PCR amplification of pRB3910 using the oligonucleotides 5′-TGTCACGGGGTCCACGCCCCCTAACATGG-3′ and 5′-CAGAAGCCCCGCCTACGTTGCGACCCCCAG-3′. The fragment was purified with the wizard PCR purification kit (Promega) and 5′ end-labeled with [32P]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 [α³²P]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 Correlate to the Donor and Acceptor Sites of Intron 1 of the aO Gene. Preliminary experiments showed that a cytoplasmic RNA hybridized to a probe containing sequences mapped in the intron 1 of the aO 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 aO gene. If the new transcript were stable aO 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 aO 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 aO intron 1 sequences. The transcripts of both 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 aO 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 aO. 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 aO gene is stable and transported to the cytoplasm. This

![Fig. 1. Schematic diagram of the domain of the aO genes. Line 1 is a linear representation of the HSV-1 genome. The unique sequences are represented as the unique long (UL) and unique short (US) 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 aO 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 aO 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 aO 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 aO intron 1 species indicated by S1 protection analyses. All four of these 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 these 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.](image-url)
RNA, designated a0 intron 1 RNA, arises from a single splice donor and several splice acceptor sites, and therefore the results predict that the a0 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 a22-/U3.5- mutant R325, we conclude that a22 and or U3.5 control the utilization of the splice acceptor site C.

The a0 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 a0 and a0 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 a0 (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 a0 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 a22-/U3.5- 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 a0 gene. The results were as follows.

(i) The intron specific riboprobe reacted strongly with broad bands corresponding to the a0 intron 1 RNA, and in small amounts, with the 2.5-kb spliced a0 transcript in extracts of HSV-1(Δ305)-infected cells (Fig. 3B, lanes 1 and 3). The a0 intron 1 RNAs were not detected with a strand-specific riboprobe antisense to exon 1 of a0 (data not shown).

(ii) The predominant species of a0 intron 1 RNAs detected in rabbit skin cells infected with the R325 virus (Fig. 3B, lane 2) migrated faster than those derived from HSV-1(F)Δ305 infected cells (Fig. 3B, lane 1).

We conclude from this series of experiments the following:

(i) The amounts of 2.5-kb mature a0 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 a0 intron 1 RNAs. These results suggest that the a0 mRNA is less stable than the a0 intron 1 RNAs.

(ii) The intron specific riboprobe reacted with a family of four cytoplasmic RNAs of approximately the size expected of the a0 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 \( \alpha_0 \) RNAs accumulating in the cytoplasm.

The Altered Mobility of \( \alpha_0 \) Introns 1 Transcripts in Extracts of Rodent Cells Infected with R325 Is Not Due to Reduced Expression of \( U_5 \) 11. Earlier studies have shown that a subset of \( \gamma \) genes exemplified by \( U_5 \) 11 is underexpressed in cells infected with the \( \alpha_{22^-}/U_3 \) 1.5\(^- \) R325 mutant. In addition, it has been shown that \( U_5 \) 11 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 \( U_5 \) 11 gene rather than deletion of the \( \alpha_{22} \) and \( U_3 \) 1.5 genes, cytoplasmic RNA from rabbit skin cells or Vero cells extracted 12 hr after infection with either the parent virus HSV-1(\( \Delta305 \)) or the R325 mutant were electrophoretically separated and hybridized to a nick-translated DNA probe specific to \( U_5 \) 11. Fig. 2C shows that while \( U_5 \) 11 levels are decreased in extracts of R325-infected rabbit skin cells, \( U_5 \) 11 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 \( U_5 \) 11 expression is more drastically reduced in the same extracts, the change in migration of the new transcript is not an effect of reduction of \( U_5 \) 11 gene expression.

\( \alpha_0 \) Introns 1 Transcripts Are Not Polyadenylated. The accumulation of \( \alpha_0 \) intron 1 mRNAs in the cytoplasm was unexpected and raised the question of whether they are polyadenylated. To answer this question, total RNA from a 150 cm\(^2\) flask of rabbit skin cells infected with either the wild-type virus HSV-1(\( \Delta305 \)) 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 \( \alpha_0 \) (Fig. 1, line 4). Fig. 4 shows that the probe detected the 2.5-kb mature spliced \( \alpha_0 \) transcript in poly(A)\(^+\) selected extracts of HSV-1(\( \Delta305 \)) infected cells, but failed to detect the \( \alpha_0 \) intron 1 transcripts. In addition, the results verified the absence or low abundance of mature \( \alpha_0 \) mRNA in rabbit skin cells infected with \( \alpha_{22^-}/U_3 \) 1.5\(^- \) virus. Therefore, the \( \alpha_0 \) intron 1 RNAs accumulating in the cytoplasm are not polyadenylated.

![Fig. 4](image_url)

**Fig. 4.** Autoradiographic image of \( ^{32}P \)-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-1(\( \Delta305 \)) virus. Each lane was loaded with poly(A)\(^+\) RNA extracted from one 150-cm\(^2\) flask. Lanes are labeled according to the virus that infected the cells.

**DISCUSSION**

It is generally accepted that the coding domain of the \( \alpha_0 \) 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 \( \alpha_0 \) 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 \( \alpha_0 \) gene is stable, nonpolyadenylated and accumulates in the cytoplasm. The intron appears to be more stable than the \( \alpha_0 \) mRNA—its by product. (ii) The electrophoretic mobility of \( \alpha_0 \) intron 1 derived from rabbit skin cells infected with the \( \alpha_{22^-}/U_3 \) 1.5\(^- \) 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 pre-ICP0 RNAs, and with which, for the sake of simplicity, we have renamed ICPO-A. The implication of this conclusion is that subpopulations of \( \alpha_0 \) 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 \( \alpha_0 \) 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 \( \alpha_0 \) bears some resemblance to LATs in several respects. Like LATs, they are not polyadenylated (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 \( \alpha_0 \) intron 1 RNA accumulating in rabbit skin cells infected with \( \alpha_{22^-}/U_3 \) 1.5\(^- \) (R325) virus is significantly smaller than the corresponding RNA derived from wild-type virus-infected cells. The change in size of \( \alpha_0 \) 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 \( \alpha_0 \) 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 ICPO-A. Thus ICPO-B, ICPO-C, and ICPO-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...
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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 a27 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. We thank A. P. W. Poon for a careful reading of the manuscript. These studies were aided by grants from the National Cancer Institute (CA47451) and the National Institute for Allergy and Infectious Diseases (AI124009).

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.

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