Herpes Simplex Virus 1 RNA-Binding Protein U₅11 Negatively Regulates the Accumulation of a Truncated Viral mRNA

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Received 6 May 1991/Accepted 7 August 1991

The U₅11 gene of herpes simplex virus 1 (HSV-1) encodes a site-specific, basic, RNA-binding protein. The viral RNA sequences bound by U₅11 protein precipitated by a monoclonal antibody hybridized to a 1.3-kb BamHI C' fragment of the HSV-1 genome. This fragment encodes a U₅11-regulated transcript which accumulates to high level in the cells infected with U₅11⁺ virus but not in cells infected with wild-type virus. This transcript, designated Δ34, is a truncated form of the mRNA encoding an essential protein encoded by the U₅34 open reading frame. The U₅11 protein was shown to bind Δ34 RNA at or near its 3' terminus. The nucleotide sequence of the region surrounding the termination of transcription of Δ34 RNA transcription suggests that the latter may be the product of transcriptional attenuation. U₅11 protein resembles the tat protein of human immunodeficiency virus with respect to size, charge, nucleolar accumulation, and possibly effect on accumulation of its target RNA but does not share with it discernible sequence homology.

In the course of an attempt to determine whether the major viral regulatory protein binds to viral RNA, we discovered, as previously reported (31), that cells infected with herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) express a site- and conformation-specific RNA-binding activity. We mapped this activity to a single viral gene designated U₅11; we further showed that U₅11 protein is the only viral gene product required for this activity and that U₅11 protein participates in the RNA/protein complex.

U₅11 protein is a relatively small, basic protein that has been reported to localize to the nucleoli of infected cells (16). In these respects, and in the possession of site- and conformation-specific RNA-binding activity, it is similar to the tat and rev regulatory proteins of human immunodeficiency virus (reviewed in reference 4). We have proposed that U₅11 protein might also act to regulate the accumulation of RNAs to which it binds. Nonetheless, several characteristics of U₅11 protein make it unusual for an HSV regulatory protein. First, the U₅11 gene is a prototype γ2 or late HSV-1 gene (9). All other such genes for which a function has been described encode structural components of the virion (29). Second, the U₅11 gene is completely dispensable for growth in several cell lines in which deletion mutants in that gene have been tested (15, 18, 21).

In our initial studies, the binding of U₅11 to RNA was demonstrated with an in vitro transcript containing sequences antisense to a portion of the U₅11 gene itself. We could not demonstrate the existence of an authentic HSV RNA transcript which contained this probe sequence, and thus no authentic infected-cell substrate for U₅11 binding was known. To identify an authentic substrate of U₅11, we took advantage of the RNA-binding activity of U₅11 protein to isolate binding substrates from HSV-1-infected cells and to investigate whether U₅11 regulated their accumulation in infected cells.

In this report, we show that U₅11 protein binds to at least one specific viral RNA substrate. We have mapped the bound RNA to a small fragment of the HSV genome and shown that this fragment encodes a transcript that is responsive to U₅11 such that it is manyfold more abundant in cells infected with mutants lacking the U₅11 gene than in cells infected with the wild-type virus. We show that this transcript is a truncated, possibly attenuated transcript of an essential viral gene which, like U₅11, is expressed late in infection.

MATERIALS AND METHODS

Cells and viruses. HeLa (American Type Culture Collection) and HEp-2 (M.A. Bioproducts) cells were propagated and infected as previously described (10, 30). The properties and propagation of HSV-1 strain F (HSV-1(F)) and the deletion mutants HSV-1(F)Δ305, R3630, and R3631 have been described elsewhere (6, 18, 24, 30). Recombinant virus R3630-R was constructed by marker rescue of R3630 viral DNA by plasmid pRB421 (18), which contains wild-type U₅11 and α47 gene sequences derived from HSV-1(F). Recombinant virus R4231 was constructed by recombination between R3630 viral DNA and plasmid pRB4231, which contains the sequences of pRB421 into which a cytomegalovirus envelope glycoprotein epitope-encoding sequence (14) was inserted at an XhoI site immediately preceding the U₅11 ATG initiator codon. The methods used for cotransfection and selection of these recombinant viruses have been described elsewhere (25, 31).

Isolation and analyses of RNA. Cytoplasmic RNA was purified as described by Jenkins and Howett (8). Fractionation of RNAs on formaldehyde-agarose gels was done as described by Maniatis et al. (17). Gels were blotted to Zeta-Probe membrane (Bio-Rad) and probed with strand-specific RNA probes, using protocols recommended by the manufacturer except that hybridization and washing of blots were carried out at 80°C. Probe for 5' and 3' end analysis were labeled with polynucleotide kinase and Klenow fragment, respectively, using standard procedures (17). S1 analyses were done as described previously (8).

Assays for RNA-binding activity. The generation and properties of a T7 RNA polymerase-transcribed probe from pRB3881 and methods for gel shift and RNase T₁ protection RNA-binding assays were as previously described (31).

Immunoprecipitation of U₅11/RNA complexes. Total cellu-
lar extracts of HSV-1(F)-infected HeLa cells were prepared by combining cytoplasmic and nuclear extracts prepared according to Lee et al. (13). The concentration of protein in the extracts was determined with a Bio-Rad protein assay kit. For immunoprecipitation, extracts were diluted to 500 µg/ml with binding buffer (31) and RNasin was added to 1,000 U/ml. Anti-U$_5$11 monoclonal ascites fluid was added to a dilution of 1:200, and the mixture was incubated at room temperature for 30 min. An equal volume of a 50% suspension of goat anti-mouse immunoglobulin G-agarose (Sigma) in binding buffer was then added, and the reaction was mixed on a rotating wheel for a further 30 min. The mixture was digested with RNase T$_1$ (Sigma) at a concentration of 3 U/µl for 10 min at room temperature and then incubated for 10 min in the presence of 5 mg of heparin (Sigma grade 1) per ml. Antibody-agarose was pelleted by a 1-s spin in a microcentrifuge, washed three times with binding buffer containing 1 mg of bovine serum albumin per ml, 500 mM NaCl, and 5 mg of heparin per ml, and then washed once more with binding buffer alone. The immune complexes were solubilized by incubation in elution buffer (0.1 M Tris [pH 7.5], 50 mM NaCl, 10 mM EDTA, 1.0% sodium dodecyl sulfate [SDS]) at 65°C for 5 min. RNA was purified from the eluate by extractions with 1:1 phenol–chloroform and with chloroform alone followed by ethanol precipitation.

**Sucrose gradient fractionations.** Infected monolayers of HEP-2 cells (5 × 10$^7$ cells) were rinsed with phosphate-buffered saline (PBS) and then scraped into 5 ml of PBS and pelleted. The cell pellet was resuspended in 4 volumes of gradient buffer (50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.4], 250 mM NaCl, 10 mM MgCl$_2$, 1 µg of cycloheximide per ml) containing 250 mM sucrose. Cells were lysed by addition of Triton X-100 to 1.0%, and nuclei were pelleted by a brief spin (20 s) at top speed in a microcentrifuge. Deoxycholate was added to the supernatant fluid to a final concentration of 1.0%, and the lysate was spun again at top speed in a microcentrifuge for 10 min. The supernatant fluid was layered on top of an 11-ml linear gradient of 0.5 to 1.0 M sucrose in gradient buffer. Samples were centrifuged at 40,000 rpm in a Beckman SW41 ultracentrifuge rotor for 6 h and decelerated without a brake. Gradients were fractionated from the top to avoid disrupting the polysomal pellet. RNA was purified from gradient fractions by the procedure of Peppel and Baglioni (23) except that just prior to isopropanol precipitation, 10 µg of glycogen was added to each sample to act as a carrier.

**Immunoblotting.** Proteins separated by SDS-polyacrylamide gel electrophoresis were electrophoretically blotted onto nitrocellulose. Reagents for probing immunoblots were obtained from Zymed Laboratories (Immunoblot SAP kit for mouse antibody). The nitrocellulose membrane was blocked using 3% gelatin in Tris-buffered saline (TBS; 20 mM Tris [pH 7.5], 500 mM NaCl), washed twice for 5 min each time in T-TBS (TBS containing 0.05% Tween 20), and then incubated with a 1:1,000 dilution of primary antibody in incubation buffer (T-TBS containing 1% gelatin). The membrane was then washed in T-TBS as before, reacted with a 1:250 dilution of biotin anti-mouse immunoglobulin G conjugate in incubation buffer for 30 min, washed, and then reacted with a 1:250 dilution of streptavidin-alkaline phosphatase conjugate in incubation buffer for 20 min. Following a final wash, color was developed by using reagents and protocols supplied with the kit.

![Image](http://jvi.asm.org/)

**FIG. 1.** Characterization of anti-U$_5$11 monoclonal antibody. (A) Photographic image of a blot of HSV-1(F)-infected cell proteins probed with anti-U$_5$11 monoclonal antibody. Reactive species are marked with arrowheads. (B) Photographic image of a blot of proteins from cells infected with R4231, which contains U$_5$11 with an N-terminal cytomegalovirus epitope insertion, probed with anti-cytomegalovirus epitope antibody. (C) Autoradiographic image of nondenaturing electrophoretic separation of the complex formed between PRP3881 T7 transcript probe and no protein (lane 1), infected cell extract protein (lane 2), anti-U$_5$11 monoclonal antibody (Ab) (lane 3), and both infected cell extract and anti-U$_5$11 monoclonal antibody (lane 4).

**RESULTS**

**Reactivity of monoclonal antibody to U$_5$11 protein.** The experimental objectives of these studies were to isolate the RNA bound to U$_5$11 by immunoprecipitation of the protein from infected cell extracts with antibody. To produce a monoclonal antibody, we immunized mice with U$_5$11/β-galactosidase fusion protein. The production and properties of this antibody will be described elsewhere (31a). The experiments described below show that monoclonal antibody cl28 reacted specifically with U$_5$11 protein and recognized U$_5$11/RNA complexes.

Figure 1A shows the reactivity of the antibody with lysates of HEP-2 cells harvested 24 h after infection with HSV-1(F), electrophoretically separated in denaturing polyacrylamide gels, and electrophoretically transferred to a nitrocellulose sheet. The antibody reacted with a pair of bands with a Mr of approximately 23,000 (upper arrowhead), which correspond to full-length U$_5$11 protein, and to a lesser extent with a set of smaller bands (lower arrowhead). Both sets of bands are derived from the U$_5$11 open reading frame, since insertion of a heterologous epitope tag into the U$_5$11 open reading frame results in the detection of both sets of bands.
in Western immunoblots probed with the corresponding antigenic antibody (Fig. 1B). The lower-molecular-weight bands may be degradation products of US11 protein.

The ability of the antibody to react with specific US11 protein/RNA complexes was tested by incubation of specific probe with US11 protein alone (Fig. 1C, lane 2), antibody alone (lane 3), or US11 protein and antibody together (lane 4), followed by electrophoresis on a nondenaturing polyacrylamide gel. As shown in the Fig. 1, the electrophoretic mobility of the complex formed between US11 protein and the RNA probe is further retarded in the presence of the antibody. The retardation of the RNA probe by the antibody was dependent on interaction with US11 protein, since in the absence of US11 protein the antibody had no effect on the migration of the probe (Fig. 1C, lane 2).

Antibody to US11 protein immunoprecipitated specific RNAs from infected cells which hybridized to the HSV-1(F) BamHI C' fragment. Total extracts from HSV-1(F)-infected HeLa cells were prepared and incubated sequentially with anti-US11 antibody, agarose-conjugated secondary antibody, and RNase T1. The digestion with RNase T1 was necessary to degrade RNA which binds adventitiously to US11 protein because of its high positive charge. The immobilized immune complexes were pelleted by centrifugation, washed in high salt, and disrupted with SDS. The RNA was purified from the eluate, labeled with polynucleotide kinase, and separated on a denaturing polyacrylamide gel. The results (Fig. 2A) show that discrete RNA species were precipitated with anti-US11 antibody (lane 4), but only from extracts of cells infected with wild-type HSV-1 and not from extracts of cells infected with R3631, a mutant in which the US11 promoter and the sequences encoding ICP47 have been deleted (18) and which does not express US11 protein (compare lanes 2 and 4). It is not clear whether the large number of bands observed in lane 4 represent completely different RNA species or resulted from partial digestion of a more limited number of species with RNase T1.

To test whether the RNA bound to US11 protein contains viral transcripts, RNase T1-resistant fragments immunoprecipitated with anti-US11 antibody and labeled with polynucleotide kinase were hybridized to an electrophoretically separated BamHI digest of HSV-1 DNA (Fig. 2B). One band, corresponding to the 1.3-kbp BamHI C' fragment (indicated by the arrowhead), hybridized faintly but reproducibly, suggesting that one or more US11 protein-binding RNAs are encoded on this fragment.

The BamHI C' fragment of HSV-1 encodes a small transcript whose abundance is regulated by US11 protein. The purpose of this series of experiments was to determine whether the US11-bound RNA that mapped to the BamHI C' fragment corresponded to a transcript regulated by US11 protein. RNAs extracted from HeLa cells infected with 5 PFU of HSV-1(F) or with the recombinant R3631 per cell and maintained for 18 h were separated on a formaldehyde-agarose gel, transferred by blotting to a Zeta-Probe membrane, and probed with labeled SP6 or T7 in vitro-generated transcripts of the BamHI C' fragment cloned in pBR176 (Fig. 3 and 4C) to detect leftward and rightward (Fig. 4B) transcription, respectively. The results of the assay for rightward transcription products are shown in Fig. 3A. Six transcripts were consistently observed in RNAs from both viruses. Three transcripts of approximately 9.2, and 1.8 kb could be tentatively assigned on the basis of size to a previously mapped read-through transcript from the HSV-1 US130 open reading frame encoding the DNA polymerase gene and transcripts of the US33 and US134 open reading frames, respectively. The remaining three could not be so assigned and were otherwise unusual in that although they accumulated in the cytoplasm, they were not retained on oligo(dT)-cellulose and were presumably nonpolyadenylated (Fig. 3B).

The smallest of the three, which we shall designate as Δ34, was reproducibly six- to eightfold more abundant in cells infected with the mutant R3631 virus than in cells infected with wild-type virus. To demonstrate that this difference between R3631 and wild-type virus in the level of this small RNA is attributable to the deletion in R3631, a recombinant virus in which those specific sequences were restored by homologous recombination with a plasmid containing wild-type sequence was constructed. RNAs were isolated from cells infected with US11 virus, US11 virus, and the US11-restored virus at 18 h postinfection and analyzed by Northern (RNA) blot (Fig. 3C). The US11-restored virus (lane 3) expressed low, wild-type levels of the US11-regulated RNA, and therefore we conclude that both the wild-type and US11-restored viruses suppress the accumulation of this RNA.

Fine mapping of the Δ34 transcript. The mapping experiments described below indicate that the Δ34 transcript is 5′
coterminal with the transcript of the U₃₄ open reading frame. Preliminary mapping experiments with restriction fragments indicated that the U₃₆-regulated transcript was contained within the domain of the U₃₄ open reading frame, that is, in the Xba-Bam fragment of BamHI C', and that it spanned the EcoRI site (data not shown). The precise localization of the sequences encoded in the Δ₃₄ RNA was done by S1 nuclease protection studies. To differentiate the 5' and 3' ends of the regulated transcript from those of other transcripts which initiate in the same region, cytoplasmic extracts of infected cells were fractionated by centrifugation through a 0.5 to 1.0 M sucrose gradient and RNA was purified from the fractions. Fractions from the top half of the gradient were first assayed by Northern blot to determine the BamHI C' transcript distribution in the gradient; these assays (Fig. 5A) showed that the U₃₆-regulated Δ₃₄ RNA peaked in fraction 3 of the gradient and was well separated from other hybridizing RNAs. The RNAs from the same fractions were then tested in two S1 assays, one using the large Eco-BamHI fragment of BamHI C' which had been 5' end labeled at the EcoRI site to detect 5' ends of RNA and another using the Eco-BsrB fragment of pRB3979 which had been 3' end labeled at the EcoRI site to detect 3' ends of RNA (Fig. 4B). The S1 assay for RNA 3' ends (Fig. 5B) showed a 226-nucleotide (nt) protected fragment which peaked at the same position as Δ₃₄ RNA and thus corresponds to the 3' end of the regulated transcript. The S1 assay for RNA 5' ends, on the other hand, showed a single protected 262-nt fragment in all of the fractions which contained RNA detected by Northern analysis (Fig. 5C), suggesting that all of the major hybridizing species in the Northern assay, including the U₃₆-regulated RNA and the U₃₄ mRNA, share the same 5' end. This 5' end is associated with a consensus TATA box (Fig. 4D). The U₃₆-regulated transcript thus appears to be a truncated form of the U₃₄ mRNA (Fig. 4B). The length of the U₃₆-regulated RNA determined from the S1 assays is 485 nt. This is consistent with the size of the transcript (about 450 nt) determined by Northern blotting using in vitro transcripts of fragments of the U₃₄ gene as size standards (not shown).

The DNA sequence in the vicinity of the 3' end of the U₃₆-regulated Δ₃₄ transcript is diagrammed in Fig. 4E. There is no polyadenylation signal, consistent with the lack of a poly(A) tail in the accumulated RNA. Neither is there a...
The Δ34 RNA sequence contains a U511-binding site. Since Δ34 RNA is regulated by U511 and is encoded in the region of the genome shown to encode a U511-bound RNA, it seemed likely that Δ34 RNA might be a substrate for U511 binding. To test this directly, sequences encoding Δ34 RNA were transcribed in vitro and used as a probe in a RNase T1 protection (binding) assay. In an initial experiment (Fig. 6A), the probe was transcribed from a DraIII-AvaI fragment containing (i) all of the Δ34 sequence except 20 nt from the 5′ end and (ii) an additional 31 nt beyond the 3′ end (Fig. 6B). This probe was incubated with proteins from uninfected (Fig. 6A, lanes 1 and 2), wild-type-infected (lanes 3 and 4), and U511−-infected (lanes 5 and 6) cells in the presence or absence of anti-U511 antibody, digested with RNase T1, and electrophoretically separated on a nondenaturing polyacrylamide gel. A heterogeneous set of labeled bands formed in the presence of wild-type-infected cell protein which were not present in uninfected or U511-infected extracts (compare lane 3 with lanes 1 and 5). The electrophoretic mobility of this set of bands was further reduced in the presence of anti-U511 antibody (lane 4), indicating that U511 protein was present in the complexes. The results of this experiment indicated that Δ34 RNA sequences contain a U511-binding site. To map the position of this binding site further, probes truncated at the 3′ or 5′ end were tested in this binding assay. As summarized in Fig. 5B, deletion of as little as 36 nt of Δ34 sequence from the 3′ end eliminated binding, but at least 257 nt could be deleted from the 5′ end without loss of the binding site. These results indicate that the U511-binding site present in Δ34 RNA sequence is at or near the 3′ end.

DISCUSSION

Functions of U511 protein in infected cells. In this report, we show that the site- and conformation-specific RNA-binding protein U511 negatively regulates the accumulation of a viral RNA called Δ34 in infected cells. We have further shown that this RNA contains a sequence at or near its 3′ end to which U511 protein can bind in vitro. We have also observed that antibody to U511 immunoprecipitates an RNA mapping at or very near the Δ34 locus from infected cell lysates, suggesting that U511 protein also binds to this RNA in vivo. These observations raise several intriguing questions: (i) Is the sole RNA whose abundance is regulated by the U511 protein? (ii) What is the significance of the regulation of Δ34? (iii) By what mechanism does the U511 protein regulate the abundance of the Δ34?

The RNAs regulated by U511 protein. Although we have identified only one viral substrate for U511 binding and regulation, the results presented in Fig. 2 suggest that the U511 protein may regulate the abundance of more than one RNA. The pattern of RNase T1-resistant RNAs immunoprecipitated from infected cell lysates with anti-U511 antibody
Formation and regulation of Δ34 RNA. Δ34 RNA, which initiates at the same site as U34 mRNA yet is considerably shorter, might form in one of four ways. (i) It may form by normal 3' end formation using an inefficient polyadenylation site. This alternative is excluded inasmuch as the Δ34 transcript is nonpolyadenylated; furthermore, there is no consensus polyadenylation site within the body of the U34 gene short of that which would give rise to full-length U34 mRNA. (ii) It may be formed by splicing of the full-length U34 mRNA. Several considerations suggest that Δ34 RNA is not a spliced form of U34 mRNA and that the 3' end detected in S1 analysis (Fig. 5B) is in fact the 3' end of the transcript. First, the length of Δ34 RNA, as judged by its mobility in denaturing gels relative to the mobilities of size standards derived from in vitro-transcribed BamHI C' sequences, is about 450 nt (data not shown), consistent with the combined size of the products protected in S1 assays. Second, the 3' end identified in the S1 assay does not match the consensus sequence for a splice donor site (22), although there is a GT dinucleotide within a few bases. Third, splicing alone is insufficient to account for the structure of Δ34 RNA, since it would result in a polyadenylated mature transcript. Either of alternatives iii and iv below would still be required to account for the data. (iii) The truncated transcript might be generated from a longer, perhaps full-length U34 mRNA by specific cleavage. The data presented here neither support nor exclude this alternative. (iv) Transcription of the U34 mRNA might terminate prematurely at the mapped 3' end. The sequence in the vicinity of the 3' end (Fig. 4E) is consistent with and indeed suggestive of this alternative in that it consists of a run of T residues embedded in a GC-rich region with secondary structure-forming potential quite similar to those of the termination sites of attenuated products of the c-myc (3), adenovirus major late (32), simian virus 40 late (28), and mouse minute virus P4 (27, 28) promoters.

U34 protein could suppress the accumulation of Δ34 RNA either by suppressing its formation or by enhancing its degradation. If, in fact, Δ34 RNA is the product of premature termination by RNA polymerase, then suppression of the formation of this RNA would be essentially equivalent to an antitermination function. Such a function has been proposed for the tat protein of human immunodeficiency virus (HIV-1, 10, 12, 33). Although U34 and tat are both small, basic RNA-binding proteins which localize to the nucleolus, there is no demonstrable homology in their amino acid sequences, and though the site(s) at which tat-regulated transcription prematurely terminates is not certain and may not be specific (33), the sequence in the vicinity of the 3' end of accumulated short transcripts (10) is at least superficially unlike that present in the U34 gene.

Significance of U34 regulation. We have not yet determined the function of Δ34 RNA and its regulation in the viral life cycle, but two possibilities present themselves. (i) Δ34 RNA might have a messenger or structural function of its own. The absence of a poly(A) tail and a termination codon for the portion of the U34 open reading frame contained within it would make Δ34 a rather unconventional mRNA. Nonetheless, it must contain any translational initiation signals present in 5' end of U34 mRNA, and it appears to associate with ribosomes (unpublished data). (ii) Δ34 RNA is a by-product of the regulation of U34 mRNA. All of the possible mechanisms of Δ34 formation noted above must occur at the expense of the formation of full-length U34 mRNA. It seems likely that in one of the natural host cell types, the formation of Δ34 RNA may occur at a much higher level than that seen in HEp-2 cells and thus signifi-
cantly decrease or even eliminate the accumulation of full-length U3.34 mRNA. This would be consistent with a premature termination mechanism similar to that operating for the c-myc, c-myb, and c-fos genes, since premature termination is most pronounced in differentiated cells (1, 2, 5, 7, 20). It is therefore possible that the balance between formation of the truncated or full-length product is a regulatory switch or modulation point for genes regulated by U3.34. The U3.34 gene appears to be essential for growth in cell culture (26), and the U3.34 open reading frame has been proposed to encode a virion component (19). To the degree that U3.11-regulated genes are, like U3.34, essential for viral replication, such regulation by U3.11 can have a large effect on the viral life cycle.

ACKNOWLEDGMENTS
We thank Lenore Pereira for the invaluable gift of monoclonal antibodies. These studies were aided by Public Health Service grants from the National Cancer Institute (CA47421) and the National Institute for Allergy and Infectious Diseases (AI12409 and AI1588-11).

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