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The Herpes Simplex Virus U_S11 Open Reading Frame Encodes a Sequence-Specific RNA-Binding Protein

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Herpes simplex virus 1- and 2 (HSV-1 and HSV-2)-infected cell extracts but not uninfected cell extracts contain an RNA-binding activity for an in vitro-transcribed sequence from the domains of the HSV-1 U_S11 and α47 genes. The transcript of this sequence has not been detected in infected cells. The binding is sequence and secondary structure specific and protects approximately 95 nucleotides from RNase digestion. Analyses of HSV-1 × HSV-2 recombinants and HSV-1 deletion mutants mapped the function necessary for activity to the U_S11 or α47 open reading frame. The α47 gene was excluded, since the RNA-binding activity is a late (γ_2) function dependent on viral DNA synthesis for its expression. The U_S11 function is the only viral function required, since translation in rabbit reticulocyte lysate of an in vitro-synthesized U_S11 mRNA resulted in the appearance of the RNA-binding activity. The product of the U_S11 open reading frame is associated with the RNA probe-protein complex inasmuch as insertion of a sequence encoding in frame 15 additional amino acids at the C terminus of the U_S11 protein caused a corresponding decrease in the electrophoretic mobility of the binding complex.

In this paper we report that a protein encoded by an open reading frame in the small component of the herpes simplex virus 1 (HSV-1) genome participates in the formation of a sequence-specific RNA-protein complex in vitro. This is the first report of an HSV protein with sequence-specific affinity for RNA. Relevant to this report are the following.

(i) The 74 known open reading frames of HSV-1 (1, 3, 5, 24, 25) form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (10, 11). Among the products of the α genes, the first group to be expressed, is the product of the α4 gene, designated as the infected cell protein no. 4 (ICP4). This protein binds to the 5′ untranscribed and transcribed noncoding domains of β and γ_1 and γ_2 genes expressed later in infection (14). Because ICP4 binds to transcribed noncoding domains of γ_2 genes, that is, genes stringently dependent on viral DNA synthesis for their expression, there existed the formal possibility that ICP4 binds to RNA as well as DNA, a possibility that is not too farfetched in light of the discovery of a host protein which binds single- and double-stranded RNA and DNA in sequence-specific fashion (36). By a fortuitous coincidence, we chose to test the transcripts of the γ_2 U_S11 (13) open reading frame. While we were unable to show that ICP4 binds to RNA, we did discover that U_S11 protein binds to a transcript complementary to its domain.

(ii) The protein product of the U_S11 gene was first identified, using hybrid arrest of translation, by Rixon and McGeoch (33) and was shown to be a protein with an M_r of 21,000. Subsequently, using an antiserum directed against a peptide sequence predicted by the nucleotide sequence of the U_S11 open reading frame, Johnson et al. (13) showed that the U_S11 protein actually consists of a doublet with an M_r of 21,000 to 22,000 and that the expression of U_S11 is regulated as a γ_2 or true late gene. Johnson et al. (13) also reported that the size of the U_S11 protein varied between two of the strains tested—an observation which had been predicted on the basis of the differences in the size of the open reading frame found in two different strains of HSV-1 (33, 43). These results were consistent with those of Lonsdale et al. (20), who had earlier shown that a doublet of proteins with an M_r of approximately 20,000 from a number of HSV-1 laboratory strains and isolates passaged a limited number of times outside the human host varied significantly in its electrophoretic mobility in denaturing polyacrylamide gels.

(iii) Our laboratory and other laboratories have reported a large number of HSV-1 genes that are dispensable for growth in cells in culture. Relevant to this report are studies demonstrating that, with one exception, all of the genes mapping in the unique sequences of the S component (U_S), including the U_S11 open reading frame, are dispensable (19). None of the studies done to date in our laboratory or reported elsewhere have shown that the U_S11^- HSV-1 strains are deficient for replication in any of the cell lines tested.

MATERIALS AND METHODS

Cells and viruses. HSV-1 strain F [HSV-1(F)], HSV-2 strain G [HSV-2(G)], and deletion mutants R325, R7023, R7039, R3630, and R3631 were described elsewhere (7, 18, 23, 27, 29, 35). HeLa cells (American Type Culture Collection) were propagated as described elsewhere (14).

Construction and cloning of plasmids. Standard procedures were used in all constructions described in this report (22). The structures of plasmids pRB3881, pRB3910, and pRB4027 and details of their construction are given in Fig. 1.

Construction of recombinant virus. The recombinant viruses used in this study (R325, R7023, R7039, R3630, and R3631) and the one recombinant virus constructed for this study were constructed by double homologous recombination between the intact DNA of a parent virus and plasmid DNA in which nonhomologous sequences and a selectable marker were sandwiched between flanking sequences whose homologs were present in the same order and orientation in the genomic DNA. The selectable marker was the HSV-1 thymidine kinase (*tk*) gene as described by Post and Roizman (29), except that the natural promoter of the *tk* gene was replaced by the α27 promoter (19). Recombinant R3630 was constructed by replacement of the α47 and U_S11 genes with

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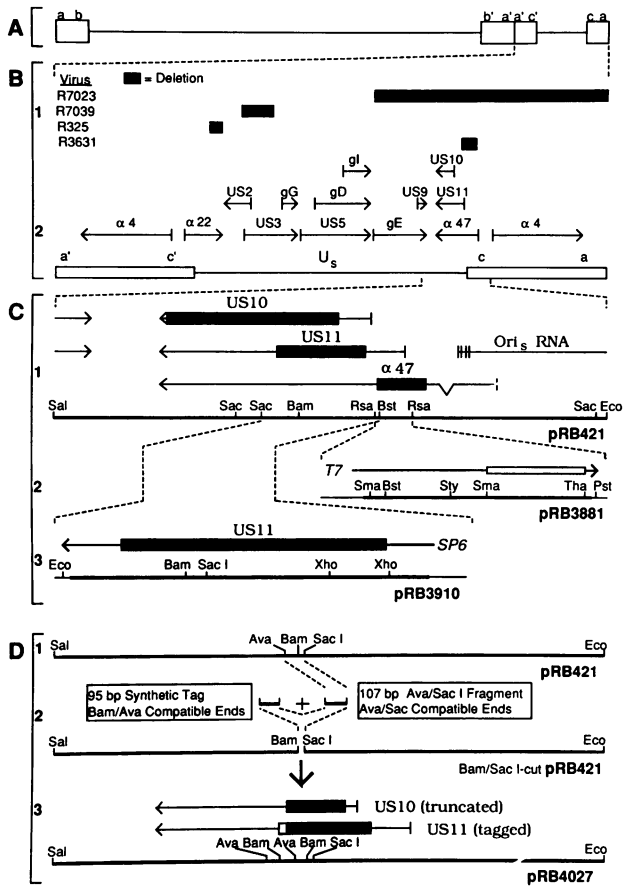

FIG. 1. Sequence arrangement of the HSV-1 genome and S component and construction of plasmids. (A) Schematic diagram of the HSV-1 genome in the prototype arrangement, showing the unique sequences (lines) flanked by inverted repeats (boxes). (B) Expansion of the S component of the genome. B1, Extent of deletions in the short component of deletion mutants used in these studies. In the genome of R7023, the S component is frozen in an inverted orientation. The deletion extends beyond the L-S junction into the inverted repeats flanking the S component. B2, Genetic map of the short component for comparison with B1. (C) Expansion of the region of the S component cloned as pRB421 and construction of plasmids pRB3881 and pRB3910. C1, Restriction and genetic maps of pRB421 (23). Vertical bars indicate transcription initiation sites; thin lines indicated transcribed noncoding domains; solid boxes indicate open reading frames α47, U_S11, and U_S10. α47 and Ori_S RNA transcripts initiate within the inverted repeat sequence; U_S11 and U_S10 transcripts initiate within U_S. Restriction enzyme abbreviations: Sac, *Sac*II; Bam, *Bam*HI; Bst, *Bst*EII; Rsa, *Rsa*I, Sal, *Sal*I; Eco, *Eco*RI. The listing of *Rsa*I sites is not complete; only the relevant sites are indicated. C2, Construction of pRB3881 probe template. The 202-nt Rsa-Rsa fragment containing 158 nt of U_S11 5′-untranslated sequence and 44 nt of 5′-flanking nontranscribed sequence was cloned into the *Hin*cII site of pUC8 and then subcloned as a *Hin*dIII-*Eco*RI fragment into pGEM-3Z. Thin lines indicate sequences transcribed by T7 RNA polymerase; the open box indicates the minimum region essential for binding (see text). For probe transcription, plasmid was linearized at the *Pst*I site and transcribed with T7 RNA polymerase to produce a 245-nt probe. Restriction sites used to generate deleted probes: Bst, *Bst*EII; Sma, *Sma*I; Sty, *Sty*I; Tha, *Tha*I; Pst, *Pst*I. 5′ deletions were made by collapsing the template between the polylinker *Eco*RI site and the appropriate site in the insert. 3′ deletions were made by linearizing pRB3881 at sites within the insert. C3, Construction of pRB3910 plasmid template for U_S11 mRNA. The 650-base-pair *Bst*EII-*Sac*II fragment of pRB421 containing the U_S11 open reading frame, 76 nt of 5′-untranslated sequence, and 90 nt of 3′-untranslated sequence was excised, converted to blunt ends by treatment with the Klenow fragment and dNTPs, and cloned into the *Sma*I site of pGEM-3Z. Linearization with *Eco*RI restriction enzyme followed by transcription with SP6 RNA polymerase produced U_S11 mRNA. Restriction site abbreviations: Eco, *Eco*RI; Bam, *Bam*HI; Xho, *Xho*I. (D) Construction of pRB4027 encoding tagged U_S11 protein. Two inserts were prepared as follows. (i) The 107-base-pair *Ava*II (Ava)-*Sac*I fragment of pRB421 (D1) encoding U_S11 codons 111 to 146 was excised and purified from a low-melt agarose gel. (ii) Synthetic oligonucleotides (94 and 95 nt) were annealed to generate an insert encoding U_S11 codons 147 to 161 followed by a sequence encoding amino acids 71 to 85 of HSV-1 ICP4 and a UAA stop codon. The annealed double-stranded insert had overhanging *Bam*HI- and *Ava*II-compatible ends. These inserts were coligated (D2) into *Sac*I/*Bam*HI-cut pRB421 to generate pRB4027 (D3). The new U_S11 open reading frame encoded a complete U_S11 plus tag. The insertion of the tag introduced a stop codon into the U_S10 open reading frame, truncating it such that only 110 codons should be translated. The construction also resulted in the duplication of the sequences between the *Bam*HI and *Ava*II sites.

the α27-*tk* gene in a homologous recombination between the genome of a *tk*-negative virus and a plasmid containing the α27-*tk* gene sandwiched between the appropriate homologous sequences. The recombinant was selected by plating the progeny of the cotransfection in medium containing methotrexate, which selects for the tk^+ progeny. Recombinant R4027 was constructed by cotransfection of intact R3630 DNA and pRB4027 plasmid DNA containing an insert in the U_S11 open reading frame as described in Results. In this instance, the *tk* gene was replaced by the HSV-1 sequences contained in pRB4027.

In vitro transcription. Plasmid templates were prepared and transcribed with SP6 and T7 RNA polymerases as recommended by Promega Biotec, except that all RNAs were capped during synthesis with the cap analog GppG (New England BioLabs) as described previously (28). Labeled binding probe was synthesized with 10 μCi of [^{32}P]CTP in a 10-μl reaction mixture supplemented with 50 μM unlabeled CTP. Reaction products were separated on denaturing 4% acrylamide urea gels and visualized by autoradiography of the wet gels. The band corresponding to full-length probe was excised, and RNA was eluted by soaking overnight in 0.5 M ammonium acetate–1 mM EDTA–0.2% sodium dodecyl sulfate. Eluted RNA was precipitated with ethanol in the presence of 10 μg of carrier glycogen and suspended in water.

Capped synthetic U_S11 mRNA for in vitro translation was transcribed from *Eco*RI-linearized pRB3910 by the same procedure, except that the concentration of all NTPs was 0.5 mM and only 0.5 μCi of [^{32}P]CTP was used as a tracer. The product was quantitated by DE-81 filter assay as described elsewhere (22).

Assays for RNA-binding activity. For gel shift assays, labeled probe (10,000 to 20,000 cpm) was treated with 5 μg of nuclear protein extract in 7 μl of binding buffer (200 mM NaCl, 20 mM Tris [pH 7.9 at 4°C], 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 20% [vol/vol] glycerol) for 20 min at room temperature, followed by addition of heparin to 5 mg/ml and reaction for a further 10 min. One microliter of loading dye (100% glycerol, 0.4% bromphenol blue, 0.4% xylene cyanole FF) was then added, and samples were applied to a nondenaturing 4% acrylamide gel poured and run in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA). Protection

assays were identical, except that before addition of heparin, reaction mixtures were digested with 5 U of RNase T_1 for 10 min.

Preparation of cellular extracts. Small-scale nuclear extracts (used for experiments shown in Fig. 5) were prepared as described by Lee et al. (16) from HeLa cells infected with 10 PFU per cell and harvested 20 h after infection. Large-scale nuclear extracts (used for all other experiments) were prepared as previously described (14).

Sephacryl S300 chromatography. One milliliter of nuclear extract was fractionated on a 55-ml column of Sephacryl S300 run in binding buffer at a linear flow rate of 8 cm/h; 0.5-ml fractions were obtained.

Preparation of competitor nucleic acids. Competitor RNAs were synthesized and purified in the same way as probe RNA, except that reaction mixtures contained all NTPs at 0.5 mM without label, and the product was excised from gels by using comigrating labeled product as a standard. Purified competitor RNAs were quantitated by A_{260}. Competitor DNAs were purified from low-melt agarose gels as described elsewhere (22) and quantitated by coelectrophoresis with DNAs of known quantity.

In vitro translation. Synthetic U_S11 mRNA (0.3 μg) was translated in a 20-μl reaction mixture by using a kit obtained from Promega Biotec according to the protocol supplied with the kit, except that after the suggested incubation period, unlabeled methionine was added to 100 μM and incubation was continued for a further 30 min. After the reaction, the mixtures were diluted to 50 μl with binding buffer and passed over a 1-ml Sephadex G-50 spin column which had been preequilibrated in binding buffer.

RESULTS

Infected-cell extracts contain an RNA-binding activity. pRB3881 (Fig. 1C) contains 5′-transcribed noncoding sequences of the γ_2 U_S11 open reading frame. Uniformly labeled in vitro transcripts of each strand of the HSV-1 sequences in pRB3881 were reacted with a partially purified preparation of ICP4 derived from Bio-Gel A.5m chromatography of crude HSV-1-infected cell nuclear extract (P. McAtee and B. Roizman, unpublished results), and the reaction mixtures were electrophoretically separated on a nondenaturing polyacrylamide gel. Only one of the RNA probes, i.e., that corresponding to the opposite strand of the U_S11 open reading frame 5′-untranslated sequence, exhibited a slower electrophoretic mobility (Fig. 2A, lanes 1 and 2). Digestion of the mixture with proteinase K either before or after the reaction (Fig. 2A, lanes 3 and 4, respectively) caused the RNA probe to move with normal mobility on the gel, showing that the retardation of the probe was due to the formation of an RNA-protein complex.

Because the degree of retardation appeared to be too small to be caused by a protein as large as ICP4 and because addition of anti-ICP4 antibody to the incubation failed to cause a further decrease in the mobility of the probe, as has been shown for ICP4-DNA complexes (14), it seemed unlikely that the retardation of the probe was due to ICP4.

RNA-binding activity is secondary structure specific. RNA probe transcribed from pRB3881 was always purified as a single tight band from denaturing polyacrylamide gels. In nondenaturing gels, however, it migrated as two species. The faster-migrating species composed 70% of the label and formed a relatively diffuse band, whereas the slower-migrating form composed 30% of the total and formed a relatively tight band (Fig. 2A, lane 1). Elution of either

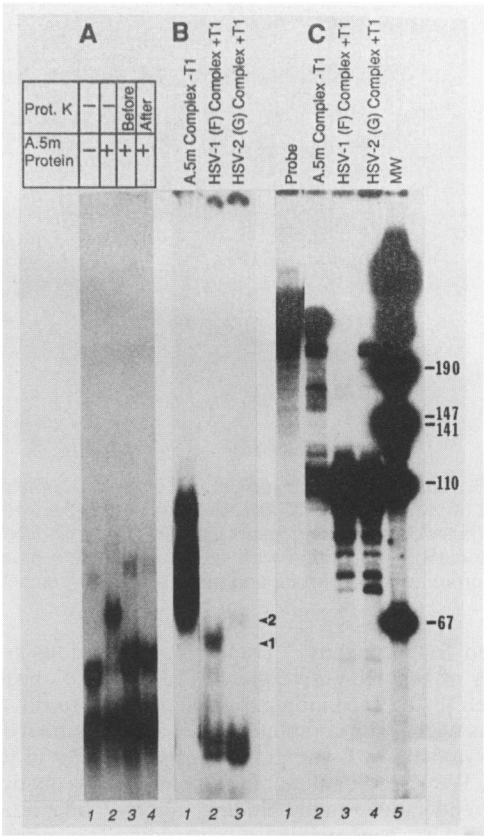

FIG. 2. Demonstration of binding to specific probe and protection of probe from RNase T_1 digestion. (A) Autoradiographic image of pRB3881 T7 transcript probe reacted as described in Materials and Methods with binding buffer (lane 1), 7 μl of Bio-Gel A.5m fraction (lane 2), 7 μl of A.5m fraction previously digested with 200 μg of proteinase K (Prot. K) per ml for 30 min at 25°C (lane 3), or 7 μl of Bio-Gel A.5m fraction subsequently digested with proteinase K (lane 4). (B) Autoradiographic image of pRB3881 T7 transcript probe reacted with 7 μl of Bio-Gel A.5m fraction (lane 1), 5 μl of HSV-1(F)-infected cell nuclear extract followed by RNase T_1 digestion (lane 2), or 5 μg of HSV-2(G)-infected cell nuclear extract followed by RNase T_1 digestion (lane 3). Arrowheads indicate the positions of specific RNase-resistant RNA-protein complexes which were excised from the gel. (C) Autoradiographic image of denaturing electrophoresis of RNAs isolated from the complexes shown in panel B. Lane 1, pRB3881 T7 transcript probe RNA; lane 2, RNA from A.5m fraction binding complex; lane 3, protected RNA fragment from HSV-1(F) binding complex; lane 4, protected RNA fragment from HSV-2(G) complex; lane 5, *Msp*I-digested, end-labeled pGEM-3Z molecular weight (MW) standards (fragment lengths shown at right).

species from gel slices followed by electrophoresis on another nondenaturing gel resulted in the reappearance of both species in the same ratio (not shown). The probe species were interconvertible and apparently represent different secondary structures adopted by the RNA probe. Only the slower species formed complexes with the infected cell proteins (Fig. 2A, lanes 1 and 2; Fig. 3B, compare lanes 1 and 2 through 14).

RNA-binding activity is sequence specific. The RNAs tested to date include transcripts of both ends of the unique short region of the HSV-1 genome and sequences from the 5′-untranslated region of the HSV-1 late gene encoding the α gene *trans*-inducing factor (αTIF). In all, several kilobases of RNA sequences have been tested, and no other sequence

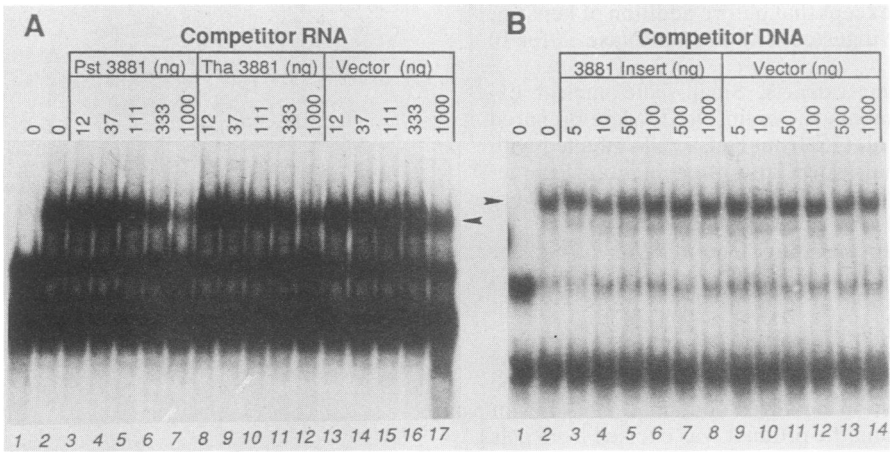

FIG. 3. Autoradiographic images of complexes formed between A.5m fraction protein and pRB3881 T7 transcript probe RNA in the presence of various competitor RNAs and DNAs. Arrowheads indicate the positions of the RNA-protein complexes. (A) Competition with specific (lanes 3 to 7) and nonspecific (lanes 8 to 17) RNA transcripts. Lane 1, Probe without protein; lane 2, probe and protein with no competitor. (B) Competition with specific (lanes 3 to 8) and nonspecific (lanes 9 to 14) double-stranded DNA competitors. Lane 1, Probe without protein; lane 2, probe and protein without competitor.

has been found to bind. Transcripts of various truncated versions of the HSV-1 sequences in pRB3881 probe have also been tested for binding. 5′ deletions up to the *Sty*I site bound as well as the complete probe, but additional deletions to the 3′-most *Sma*I site abolished the capacity of the RNA to bind. We have been unable to delete any sequences from the 3′ end and retain binding ability; linearization of pRB3881 with *Tha*I yielded a nonbinding transcript shorter by only 7 nucleotides (nt). The distance between the 3′-most *Sma*I site and the *Tha*I site is 87 nt, showing that the smallest fragment capable of binding is at least this size.

Sequence specificity of the RNA-binding activity was also demonstrated in competition assays (Fig. 3A). Specific probe was mixed with various amounts of unlabeled competitor RNAs, either specific (Fig. 3A, lanes 3 to 7) or nonspecific (Fig. 3A, lanes 8 to 17). The nonspecific competitors used were (i) the T7 transcript of *Tha*I-linearized pRB3881 and (ii) a 269-nt T7 transcript of the promoter-containing *Pvu*II fragment of pGEM-3Z which also was nonbinding in the mobility shift assay. The salient features of the result were as follows. First, the specific competitor was most effective, but all of the competitors decreased binding to some extent, and indeed it appears that the specific competitor is only three- to fivefold better than the others. The small difference may be deceptive in that only a fraction of the 3881 competitor is competitive because of secondary structure. The two nonspecific competitors, on the other hand, formed single, discrete bands in nondenaturing gels and may be completely competitive. If this is taken into account, the specific competitor was 10- to 20-fold more effective. Second, a large mass excess of specific competitor (about 300-fold) was required before competition was observed. The reason for this is unclear, since labeled probe was apparently in excess over binding activity (i.e., only a little more than half of the labeled probe was bound in the absence of competitor).

Competitions were also done with both specific and nonspecific double-stranded DNA to determine whether the activity is RNA specific. Neither the 240-base-pair *Eco*RI-*Pst*I insert fragment of pRB3881 (specific competitor) nor the 379-nt *Pvu*II fragment of pGEM-3Z (nonspecific competitor) competed effectively for the RNA-binding activity (Fig. 3B).

RNA-binding activity is present only in infected cells. Nuclear extracts from infected and uninfected cells were chromatographed on a Sephacryl S300 gel filtration column, and column fractions were assayed for RNA-binding activity by gel mobility shift (Fig. 4). RNA-binding activity was present in fractions of infected cell extract (Fig. 4A), peaking near the void volume and tailing well into the column. This activity was absent in all fractions of uninfected cell extract (Fig. 4B), indicating that some function necessary for this activity is either induced or encoded by HSV-1.

Analyses of RNA-binding activity in crude nuclear extracts. The gel shift assay described above did not permit assay of RNA-binding activity in crude nuclear extract, since competing nonspecific binding (e.g., by heterogeneous nuclear ribonucleoproteins) masked the shift. We found that it was possible to assay the RNA-binding activity in crude extract by a protection assay as described by Leibold and Munro (17), in which the binding reaction was followed by digestion with RNase T_1, which destroyed nonspecific complexes. Mixtures of probe and either A.5m fraction (Fig. 5A, lane 4) or crude HSV-1-infected cell nuclear extract (Fig. 5A, lane 5) resulted in the formation of two complexes between protein and protected RNA which were not present either in the absence of protein (Fig. 5A, lane 2) or in the presence of mock-infected cell protein (Fig. 5B, lane 1), confirming that the binding and protecting activity is either induced or encoded by HSV. The amount of the upper complex varied from one experiment to the next, and it was occasionally absent (e.g., Fig. 5B, lane 3). Bands seen even in the absence of protein presumably resulted from self-protection of the probe due to secondary structure. HSV-2(G)-infected cell extracts also reacted with the RNA probe and yielded two specific complexes (as with HSV-1, only the faster-migrating complex was invariably present) which migrated slightly more slowly than their HSV-1 counterparts (Fig. 5B, lane 2).

RNA-binding activity requires the product of the U_S11 open reading frame. In preliminary experiments, HSV-1 × HSV-2 intertypic recombinants were used to map the viral gene responsible for the mobility difference between the HSV-1 and HSV-2 complexes to the unique sequences of the S component. We then tested four deletion mutations in genes mapping in the S component (Fig. 1B). Nuclear extracts of

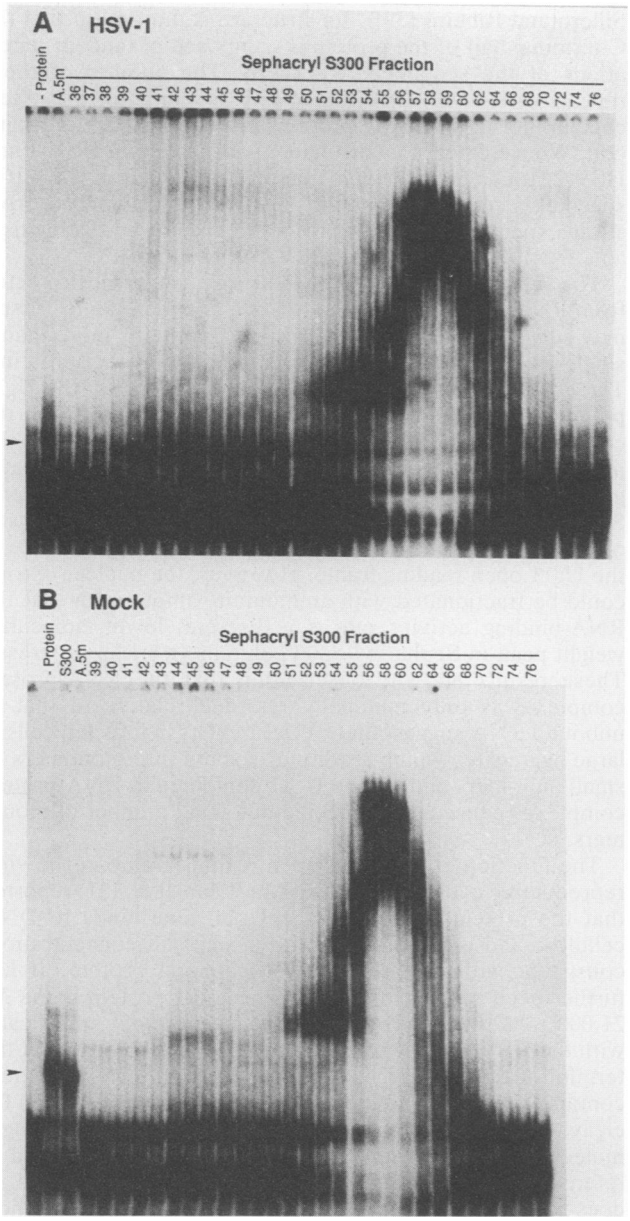

FIG. 4. Autoradiographic images of complexes formed between pRB3881 T7 transcript probe and Sephacryl S300 fractions of HSV-1(F)-infected cell nuclear extract (A) or mock-infected cell nuclear extract (B). The position of RNA-protein complex migration was determined by running a complex formed between the probe and the A.5m fraction (marked with arrowheads) in panels A and B (lanes labeled A.5m). Panel B, lane S300, shows complexes formed between probe and protein pooled from fractions 39 to 49 of the fractionation depicted in panel A.

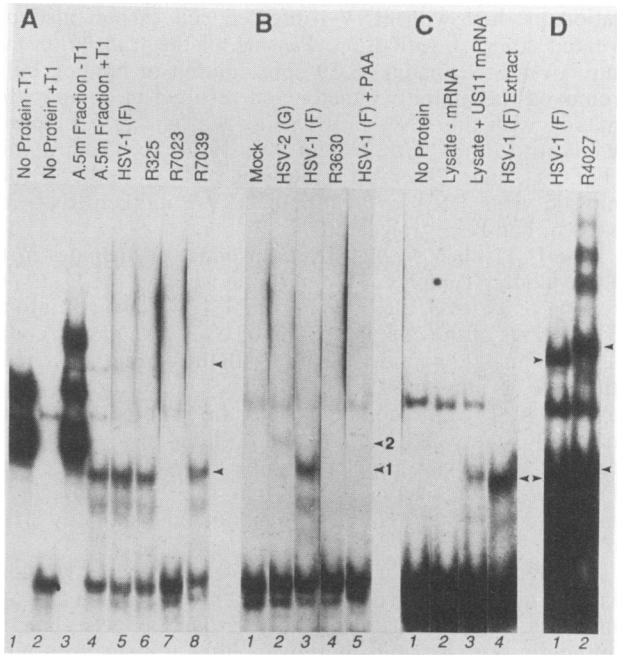

FIG. 5. Autoradiographic images of complexes formed between pRB3881 T7 transcript probe and various protein samples in gel shift (panel A, lanes 1 and 3) and RNase T_1 protection assays (all other lanes). The presence of protein or state of infection of the cells from which extract was prepared is indicated above each lane. The genes deleted in viral mutants R325, R7023, R7039, and R3631 are shown in Fig. 1B. Viral mutant R3630 carries the same deletion as R3631 but is *tk* negative. (A) Positions of the HSV-1-specific protected complexes are indicated by arrowheads. Note that although all lanes are from the same autoradiograph, only lanes 1 to 4 were adjacent in the original. (B) Positions of the HSV-1- and HSV-2-specific complexes are indicated by arrowheads marked 1 and 2, respectively. The lanes were not adjacent in the original autoradiograph. PAA, Phosphonoacetic acid. (C) Lane 1, no protein; lane 2, protein from rabbit reticulocyte lysate in which no exogenous mRNA was translated; lane 3, protein from rabbit reticulocyte lysate in which pRB3910 SP6-transcribed U_S11 mRNA was translated; lane 4, protein from HSV-1(F)-infected cell nuclear extract. The position of the binding complex is indicated with an arrowhead. The lanes were not adjacent in the original autoradiograph. (D) Comparison of complexes formed in extracts of HSV-1(F)- and R4027-infected cells. Positions of the HSV-1(F)- and R4027-specific complexes are indicated by arrowheads.

two of these, mutants R7023 (Fig. 5A, lane 7) and R3630 (Fig. 5B, lane 4), failed to bind the RNA probe. The common property of both mutants is the absence of α47 and U_S11 open reading frames.

To determine which of these two open reading frames is required, we took advantage of the observation that U_S11, a γ_2 gene, requires for its expression viral DNA synthesis and is not expressed in the presence of phosphonoacetate, an inhibitor of DNA synthesis, whereas α47 is expressed in the presence of this inhibitor. Since extracts of cells infected and maintained in the presence of phosphonoacetate did not bind the RNA probe (Fig. 5B, lane 5), we conclude that the RNA-binding activity requires U_S11.

The U_S11 open reading frame product is the only viral protein required for RNA-binding activity. In vitro transcription-translation was used to demonstrate that the U_S11 function is the only viral function required for the formation of the RNA-protein complex. In vitro transcription of *Eco*RI-linearized pRB3910 (Fig. 1C) with SP6 polymerase produced a 710-nt mRNA. When translated in rabbit reticulocyte lysate, this mRNA produced a protein which migrated in denaturing polyacrylamide gel electrophoresis as a doublet with an M_r of approximately 22,000 to 23,000 (not shown), as expected for U_S11 protein (13). In an initial experiment, incubation of binding probe with lysate in which U_S11 had been translated did not result in the formation of any specific complex. This proved to be due to an inhibitory factor in the translation mix, since mixing the crude trans-

lation product with HSV-1-infected cell extract also prevented complex formation. Passage of the translation mixture over a Sephadex G-50 spin column in binding buffer removed the inhibitory factor and resulted in complex formation with the RNA probe (Fig. 5C, lane 3). Complexes were not formed with protein from lysate in which nothing (Fig. 5C, lane 2) or an irrelevant mRNA (either Brome mosaic virus RNA or synthetic HSV-1 αTIF mRNA; not shown) had been translated.

The U_S11 open reading frame product participates in the RNA-binding complex. To determine whether the U_S11 protein participates in the binding complex, we inserted into the 3′ terminus of the coding domain of U_S11 a sequence coding a 15-amino-acid peptide in frame with the coding sequence of the gene, as illustrated in Fig. 1D. The chimeric gene, cloned as pRB4027, was recombined into the genome of recombinant R3630 in which the α47 and portions of the U_S11 open reading frame were replaced by the thymidine kinase gene. Previous studies have shown that the 15-amino-acid sequence contains an epitope for a monoclonal antibody H1091 to ICP4 (12).

The recombinant virus (R4027) selected for the thymidine kinase minus phenotype expressed a protein which migrated more slowly in denaturing polyacrylamide gels than the authentic protein, and, moreover, after transfer to a nitrocellulose sheet, the protein reacted with the monoclonal antibody H1091 (not shown). Extracts of cells infected with R4027 bound to the RNA (Fig. 5D, lane 2), and, moreover, the mobilities of the T_1-resistant complexes were slightly less than those of complexes from HSV-1(F)-infected cells, consistent with the slightly larger size of the tagged U_S11 protein. This strongly suggests that the RNA-protein complex contains U_S11 protein.

RNA-binding activity protects a large piece of the probe from RNase digestion. The deletion studies described above suggested that the minimum RNA probe size was in excess of 87 nt. It seemed unlikely that each of these 87 or more nt is required for recognition by U_S11 and, given the size of the U_S11 protein, it seemed doubtful that this entire sequence could be in contact with the protein. To test the latter idea, we determined the size of the RNA fragment protected from RNase T_1 digestion by HSV-1 and HSV-2 RNA-binding activity. The RNA probe was incubated with HSV-1(F)-infected cell nuclear extract and HSV-2(G)-infected cell nuclear extract, digested with RNase T_1, and electrophoretically separated in a nondenaturing polyacrylamide gel along with undigested probe-A.5m protein complex (Fig. 2B). Binding complex bands (Fig. 2B; indicated by arrowheads) were excised from the gel, and RNA was purified and subjected to electrophoresis on an 8% denaturing urea-polyacrylamide gel along with intact probe and molecular weight standards. The results of RNase T_1 digestions (compare Fig. 2B, lane 2, with lanes 3 and 4) indicated that HSV-1 and HSV-2 RNA-binding activities protected fragments of identical size and that the fragments were 95 to 100 nt in size.

DISCUSSION

Our studies indicate that U_S11 encodes a protein which participates in and is necessary for sequence-specific binding to an in vitro RNA transcript of an HSV-1 DNA sequence. Several aspects of these results require further comment.

The U_S11 open reading frame encodes a protein predicted to have a translated M_r of approximately 17,800. The predicted amino acid composition is that of an extremely basic protein with a predicted pI equal to 11.1 (by the method of Sillero and Ribeiro [39]). Its structure is unusual in that the C-terminal half of the protein is composed of tandem reiterations of the sequence Arg-X-Pro. The number of these repeats varies (33, 42) and probably accounts for the difference in the sizes of U_S11 proteins of different HSV-1 strains (20). We were perhaps fortunate in our choice of HSV-1 and HSV-2 strains for intertypic mapping inasmuch as HSV-1(F) specifies a relatively small U_S11 (20) and complexes from strains specifying a larger protein might not be distinguishable from those formed by HSV-2(G).

The relatively large size of the probe fragment protected from RNase digestion by the binding complex (95 to 100 nt) may suggest that the binding complex contains more than a single molecule of U_S11 and perhaps contains other proteins. The observation that a small change in the size of the U_S11 polypeptide produced a significant mobility shift in the complex, however, suggests that U_S11 is likely to be the major protein constituent of the complex. The chromatographic properties of the RNA-binding activity on Sephacryl S300 (activity peaks in the void volume) were characteristic of a protein much larger than that predicted from the size of the U_S11 open reading frame. However, the nuclear extract could be fractionated with ammonium sulfate such that the RNA-binding activity ran as a discrete, lower-molecular-weight peak in Sephacryl S300 columns (work in progress). These results and the observation that the RNA-protein complex was only minimally retarded relative to that of unbound RNA suggest that (i) U_S11 exists in infected cells in large aggregates which readily dissociate into monomers or small multimers and (ii) the U_S11 contained in RNA-protein complexes consists of a specific but small number of monomers.

The function of the U_S11 open reading frame in the viral reproductive cycle is unknown. MacLean et al. (21) reported that the protein product of the U_S11 gene binds to DNA cellulose and is eluted only with a high salt concentration, consistent with a role as a DNA-binding protein. It has further been suggested (21) that the U_S11 protein is the M_r 21,000 to 22,000 γ protein reported to interact specifically with the HSV *a* sequence (6). This sequence is located at the termini of the S and L components of HSV DNA (41) and contains several *cis*-acting sites, including those required for cleavage of concatemers of viral DNA into unit-length molecules and packaging of the viral genome (reviewed in reference 34). Several lines of evidence suggest that U_S11 does not play a role in cleavage and packaging and does not, in fact, bind viral DNA in vivo. (i) The U_S11 open reading frame can be deleted from the genome with no apparent ill effects for virus growth in cell culture (18, 23). (ii) A detailed study of the factors which bind to the HSV-1 *a* sequence (4) showed no evidence of binding of U_S11 protein. (iii) Antibody localization experiments (21) show that U_S11 protein is distributed throughout both the nucleus and the cytoplasm of infected cells and is concentrated in the nucleolus. This distribution is more consistent with affinity for RNA than with affinity for DNA inasmuch as viral DNA is excluded from the nucleolus (30, 32) and in cytoplasm is present only in capsids (34). It is not, however, surprising that an extremely basic protein such as U_S11 might bind nonspecifically to DNA, or any other polyanion for that matter, in vitro.

The secondary structure specificity of U_S11 binding might have one of two general causes. It may be that the binding site can be recognized only in single-stranded form, and in nonbinding RNA isomers this site exists at least partly in a stem-loop structure. Alternatively, binding by U_S11 may

require the presence of a particular secondary structure element not present in the nonbinding forms of the probe. It is formally possible that U_S11 recognizes some secondary RNA structure element(s) regardless of its sequence.

The probe to which U_S11 specifically bound in these experiments is complementary to the α47 and U_S11 mRNAs. The sequences protected by the RNA-binding activity are not present in any of the previously mapped transcription units, and sensitive RNAse protection assays on infected cell RNA failed to detect a corresponding transcript (work in progress). These observations suggest two mutually exclusive alternatives. First, the U_S11 RNA-binding activity may act as a negative regulator of RNA sequences to which it binds, and the probe sequences may be transcribed but fail to accumulate in significant amounts in cells which express U_S11. To test this hypothesis, we must construct a virus in which the U_S11 RNA-binding activity is not expressed but in which the probe sequences and surrounding sequences are not grossly disrupted. None of the U_S11^- deletion mutants meet this requirement.

Second, the probe sequences used here may not be transcribed in vivo and are merely a substrate analog. While this is an attractive hypothesis, the observation that the 5′ transcribed noncoding domain of the gene contains the target sequences of the gene product is in fact too much of a coincidence. To probe this hypothesis further, we have RNA affinity studies for the U_S11 RNA-binding activity in progress.

The function of the product of the U_S11 open reading frame is not known, but several possible functions which could result from binding of RNA late in infection have been excluded. Specifically, (i) host protein synthesis is decreased grossly late in infection by a viral function other than that of the *vhs* gene (encoded by the U_L41 open reading frame), which degrades RNA upon entry of the virus into the cell (8, 15, 31). U_S11 is not involved in the late degradation of host RNA inasmuch as the cells infected with the wild-type parent and the α47-U_S11 deletion mutant R3631 accumulate equivalent amounts of cellular (β-actin) and viral (αTIF and glycoprotein C) mRNAs (data not shown). (ii) HSV encodes several transcripts, including those encoding the single-stranded DNA-binding protein (U_L29) (9), the U_L38 open reading frame (2), and possibly thymidine kinase (9), which terminate at more than one polyadenylation signal, and HSV-1 encodes or induces a factor which increases the efficiency with which at least one of these signals is used (26). That U_S11 is not a necessary component of this factor is suggested by the equivalent use of the U_L29 and U_L38 polyadenylation signals in HSV-1(F)- and R3631-infected cells (work in progress). (iii) Immunohistochemical studies (21) showed that U_S11 is associated with the nucleolus of the infected cell, suggesting a possible association with ribosomes. HSV infection has a profound effect on ribosomal RNA metabolism (42), and a viral protein with sequence-specific affinity for ribosomal RNA could play a role in the modulation of translation or turnover of specific mRNAs; components of a cellular system which differentially degrades mRNAs have been found to be ribosome associated (37, 38). While no differences between cells infected with the parent and R3631 mutant have been noted, experiments on this point are still in progress.

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