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Structure and Function in the Herpes Simplex Virus 1 RNA-Binding Protein U_S11: Mapping of the Domain Required for Ribosomal and Nucleolar Association and RNA Binding In Vitro

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The herpes simplex virus 1 U_S11 protein is an RNA-binding regulatory protein that specifically and stably associates with 60S ribosomal subunits and nucleoli and is incorporated into virions. We report that U_S11/β-galactosidase fusion protein expressed in bacteria bound to rRNA from the 60S subunit and not the 40S subunit. This binding reflects the specificity of ribosomal subunit association. Analyses of deletion mutants of the U_S11 gene showed that specific RNA binding activity, nucleolar localization, and association with 60S ribosomal subunits were found to map to the amino acid sequences of the carboxyl terminus of U_S11 protein, suggesting that these activities all reflect specific binding of U_S11 to large subunit rRNA. The carboxyl-terminal half of the protein consists of a regular tripeptide repeat of the sequence RXP and constitutes a completely novel RNA-binding domain. All of the mutant U_S11 proteins could be incorporated into virus particles, suggesting that the signal for virion incorporation either is at the amino-terminal four amino acids or is redundant in the protein.

The program of viral gene expression in cells infected with herpes simplex virus is regulated at multiple levels. In addition to transactivating proteins that regulate viral transcription, the virus encodes products, including the α protein ICP27, the virion host shutoff function, and the U_S11 protein, that regulate mRNA accumulation at points following transcription initiation (8, 9, 12, 17, 29). The U_S11 protein of herpes simplex virus 1 (HSV-1) is a γ_2, or true-late, protein and is among the most abundant viral proteins present in cells late in infection (10, 13). Since viruses that fail to express U_S11 protein have not been found to be impaired in cultured cells or in mice, its function is not clear (16, 18, 19).

Multiple biochemical functions have been reported for U_S11 protein. We have presented evidence that U_S11 protein is a site- and conformation-specific RNA-binding protein and that it binds to at least two RNA substrates in vivo or in vitro (27, 26). The first of these substrates is an in vitro transcript derived from the U_S11 gene itself but running antisense to the U_S11 mRNA. No RNA containing these sequences has been detected in infected cells in culture, and the functional significance of this binding is unclear. The second substrate is the HSV-1 Δ34 mRNA, a truncated form of the U_L34 mRNA. U_S11 protein binds at or near the 3′ end of this RNA and suppresses its accumulation during infection. The Δ34 RNA 3′ end is similar to other reported sites of premature transcription termination, suggesting that Δ34 is a product of transcription termination on the U_L34 template. We have proposed that U_S11 may regulate this termination event (26).

In addition to its regulatory association with Δ34, U_S11 protein makes at least three other significant associations in the infected cell. (i) Late in infection, U_S11 protein is found in both the cytoplasm and the nucleus, and nuclear U_S11 protein has been shown by immunoelectron microscopy and by immunofluorescence to concentrate in the nucleoli, suggesting some affinity for one or more components of the ribosome synthesis and assembly machinery (14, 23, 28). (ii) We have previously shown that U_S11 protein in cytoplasmic extracts of the infected cell cosediments with 60S large ribosomal subunits and with intact ribosomes and polysomes (28). (iii) U_S11 protein is incorporated into the tegument of the virus particle, presumably by specifically interacting with another protein component of the virion. This association is responsible for the recruitment of approximately 600 copies of U_S11 protein into each virion. The U_S11 protein delivered to newly infected cells in this fashion also becomes associated with ribosomes (28).

The association of U_S11 protein with the large ribosomal subunit and with nucleoli suggests that one of the large subunit rRNAs might be a substrate for U_S11 binding, and that this binding event might mediate the interaction between U_S11 protein and the ribosome. In this report, we present two types of evidence in support of this hypothesis. First, we show that β-galactosidase/U_S11 fusion protein expressed in bacteria binds specifically to purified large subunit rRNA. Second, studies on a series of U_S11 deletion mutants show that binding to RNA, association with ribosomes, and localization in nucleoli map to the same domain of the U_S11 protein and are thus likely to be manifestations of the same activity.

MATERIALS AND METHODS

Cells and viruses. HeLa cells, 143B (thymidine kinase-minus) cells, and HEp-2 cells (all from the American Type Culture Collection) were maintained as previously described (11, 21, 25). The properties of HSV-1(F), the thymidine kinase-minus recombinant HSV-1(F)Δ305, and the ICP47$^-$, U_S11^- recombinant R3630 have been previously described (7, 16).

Construction of U_S11 mutant plasmids. The structures of the plasmids used for construction of recombinant viruses and for transfection assays are shown in Fig. 1.

Carboxyl-terminal deletion mutants were generated by restriction endonuclease digestion of plasmid-borne copies of the U_S11 followed by insertion of linker sequences that contain in-frame stop codons. MutA/421 (Fig. 1, line 2), in which the U_S11 protein is truncated at amino acid 124, was constructed by cleaving the U_S11 coding sequence in pRB421 (Fig. 1, line 1) (16) with *Bam*HI, filling the 5′ overhang with Klenow enzyme and deoxynucleoside triphosphates, and inserting a *Spe*I linker with the sequence CTAGACTAGTCTAG (New England Bio-

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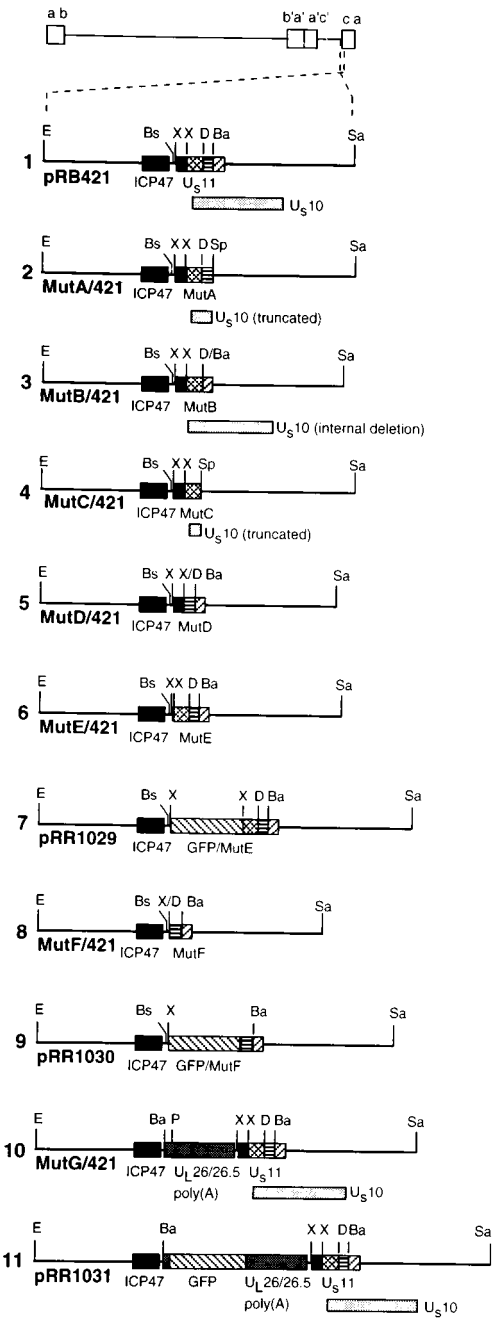

FIG. 1. Sequence arrangement of the HSV-1 genome and of plasmids used for construction of U_S11 deletion mutants and transfection experiments. A schematic diagram of the HSV-1 genome in prototype arrangement, showing the unique sequences (lines) flanked by inverted repeats (boxes), is shown at the top. Line 1 shows an expansion of the region of the HSV-1 genome cloned as pRB421, indicating the positions of restriction sites used in the construction of U_S11 deletion mutants and of the three complete open reading frames encoding ICP47 (filled box), U_S11 (multiple pattern box), and U_S10 (light stippled box below U_S11). The U_S11 open reading frame is indicated with four different fill patterns, each indicating a sequence region deleted in one or more of the mutants. Lines 2 to 11 show the sequence arrangements of the mutant plasmids used for virus construction and for transfection experiments. In pRB4526 (line 2), the U_S11 and U_S10 reading frames are truncated by insertion of an *Spe*I linker into the *Bam*HI site, yielding the mutant A (MutA) U_S11. In pRB4776 (line 3), the sequences between the *Dra*III and *Bam*HI sites are replaced with a short adaptor that maintains the reading frame, regenerating both sites and yielding the MutB U_S11. In pRB4777 (line 4), the U_S11 and U_S10 reading frames are truncated by insertion of an *Spe*I linker into the *Dra*III site, yielding the MutC U_S11. In pRB4778 (line 5), sequences between the rightmost *Xho*I site and *Dra*III site have been replaced with a short adaptor that maintains the reading frame, regenerating both sites and yielding the MutD U_S11. This deletion removes the initiation codon for the U_S10 reading frame. In pRB4779 (line 6), sequences between the two *Xho*I sites have been replaced with a short adaptor that supplies an in-frame initiation codon for U_S11, regenerating both sites and yielding the MutE U_S11. This deletion removes the probable TATA box and transcription initiation site (24) for the U_S10 gene. In pRR1029 (line 7), sequences between the two *Xho*I sites are replaced with a PCR-amplified fragment containing the complete GFP coding sequence flanked by restriction sites that permit its fusion in frame to the remainder of U_S11, yielding the GFP-MutE U_S11. In pRB4780 (line 8), sequences between the leftmost *Xho*I site and the *Dra*III site are replaced with a short adaptor that supplies an in-frame initiation codon for U_S11, regenerating both sites and yielding the MutF U_S11. In pRR1030 (line 9), sequences between the leftmost *Xho*I site and the *Dra*III site are replaced with a PCR-amplified fragment containing the complete GFP coding sequence flanked by restriction sites that permit its fusion in frame to the remainder of U_S11, yielding the GFP-MutF U_S11. In pRB4770 (line 10), a fragment containing the 3′ processing signals for the $U_L26/26.5$ gene is inserted into the *Bst*XI site downstream of the termination codon for ICP47 and just upstream of the initiation codon for U_S11. In pRR1031 (line 11), the GFP coding sequence is inserted into the *Pml*I site of pRB4770 (line 10). Restriction enzyme abbreviations: E, *Eco*RI; Bs, *Bst*XI; X, *Xho*I; D, *Dra*III; Ba, *Bam*HI; Sa, *Sal*I; Sp, *Spe*I; P, *Pml*I. The indicated *Dra*III site is not unique in these sequences.

Labs). Mutant C, in which the U_S11 protein is truncated at amino acid 88, was initially constructed in pRB3910, which contains the U_S11 protein coding sequence on a *Bst*EII-*Sac*II fragment (27), and then transferred to pRB421 to provide flanking sequences for recombination into the virus genome. pRB3910 was cleaved with *Dra*III, the 3′ overhang was removed with T4 DNA polymerase, and the same *Spe* linker used for construction of Mutant A was inserted. The *Bam*HI-*Bst*XI fragment of the plasmid that spans the deletion was then transferred to pRB421 to generate MutC/421 (Fig. 1, line 4).

Internal deletions of U_S11 were generated by replacement of restriction fragments either with small oligonucleotide adaptors that maintain the reading frame or with a fusion protein partner. These mutations were initially constructed in pRB3910 and then transferred to pRB421 to provide flanking sequences for recombination into the virus genome and for transient expression. Mutant B, in which amino acids 91 to 121 are deleted from U_S11, was constructed by cleaving the U_S11 coding sequence in pRB3910 to completion with *Dra*III, and partially with *Bam*HI, and inserting into the large partial digestion fragment a small double-stranded adaptor made by annealing two oligonucleotides having the sequences GTGTTAGG and GATCCCTAACACGCG. Mutant D, in which amino acids 37 to 87 are deleted from U_S11, was constructed by cutting pRB3910 to completion with *Dra*III, and partially with *Xho*I, and inserting into the large partial digestion fragment a double-stranded adaptor made of two annealed oligonucleotides having the sequences TCGAGGAACACCGC and GTGTTCC. Mutant E, in which amino acids 5 to 35 are deleted from U_S11, was constructed by digesting pRB3910 to completion with *Xho*I and inserting into the large fragment a double-stranded adaptor made of two annealed oligonucleotides having the sequences TCGAGATGAGCCAGAC and TCGAGTCTGGCTCA TC. Mutant F, in which amino acids 5 to 87 are deleted from U_S11, was constructed by cutting pRB3910 to completion with *Dra*III and *Xho*I and inserting into the large fragment a double-stranded adaptor made of two annealed oligonucleotides having the sequences TCGAGATGAGCCAGCCGC and GCTGG CTCATC. In each case, the mutant structure was confirmed by sequencing, and then the *Bam*HI-*Bst*XI fragment of each mutant plasmid that spans the mutation was transferred to pRB421 to generate plasmids MutB/421 (Fig. 1, line 3), MutD/421 (Fig. 1, line 5), MutE/421 (Fig. 1, line 6), and MutF/421 (Fig. 1, line 7).

pRR1029 (Fig. 1, line 7) was constructed by digesting mutant E with *Xho*I and ligating in a PCR-amplified fragment of plasmid pTUGS (gift of Stanley Perlman), which contains the *Aequorea victoria* green fluorescent protein (GFP) coding sequence (4, 22). The PCR primers used were supplied with *Xho*I cleavage sites to allow in-frame joining to the U_S11 protein coding sequence. pRR1030 (Fig. 1, line 9) was constructed by ligation of a GFP-containing PCR-amplified insert into *Xho*I-cut MutF/421.

MutG/421 (Fig. 1, line 10) was constructed by ligating the *Eco*RI-*Hin*dIII fragment of pRB1028, containing the 3′ processing signals for both the gB and $U_L26/26.5$ genes (20), into the *Bst*XI site of pRB421. This site, present in HSV-1(F) but not in the reported sequence of the HSV-1 strain 17 syn+ U_S component, lies between the termination codon of the ICP47 protein coding sequence and the U_S11 initiation codon. The inserted fragment was oriented such that the 3′-end processing signals of the $U_L26/26.5$ gene served the U_S11 and α47 genes. pRR1031, in which the GFP coding sequence is transcribed from the U_S11 promoter/regulatory signals, was constructed by insertion of a *Kpn*I-*Eco*RI fragment of pTUGS containing the entire GFP coding sequence into the *Pml*I site of MutG/421 just upstream of the 3′ processing signals of the $U_L26/26.5$ gene.

Construction of recombinant mutant viruses. Mutant plasmids linearized with restriction enzyme *Hin*dIII were cotransfected with virus DNA from the recombinant HSV-1 R3630. Recombinant viruses in which the thymidine kinase gene

was deleted by homologous recombination were selected by growth in the presence of bromodeoxyuridine as described previously (21, 27).

Purification of ^{32}P-labeled ribosomal subunit RNAs. Approximately 2×10^8 HeLa cells were labeled by incubation for 6 h in Eagle's minimal essential medium without phosphate and then for 12 h in the same medium supplemented with 100 μCi of $^{32}P_i$ per ml. Cells were then incubated in medium containing 250 μM puromycin for 30 min to dissociate polysomes, and cytoplasmic extracts were prepared. Cell monolayers were washed twice with phosphate-buffered saline (PBS), scraped into ice-cold PBS, and then pelleted at 2,000 × *g* for 5 min. Cells were resuspended in 0.5 ml of cold sucrose gradient buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 250 mM sucrose, 80 mM KCl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol). Cells were lysed by addition of Triton X-100 to a final concentration of 1%, mixed gently for 30 s, and then centrifuged for 30 s at top speed in an Eppendorf microcentrifuge to pellet nuclei. Deoxycholate was added to a final concentration of 1% to the postnuclear supernatant to disrupt microsomal membranes, and the extract was then clarified by centrifugation in the microcentrifuge for 10 min. The clarified cytoplasmic extract was layered on top of an 11-ml linear 0.5 to 1.0 M sucrose gradient prepared in gradient buffer. The gradient was centrifuged for 6 h at 40,000 rpm at 4°C in a Beckman SW41 rotor to resolve ribosomal subunits. The gradient was fractionated into 60 fractions, and the radioactivity in each fraction was determined by Cerenkov counting. Fractions containing large and small ribosomal subunits were pooled separately, diluted to 5.0 ml with gradient buffer, and then centrifuged for 15 h at 48,000 rpm at 4°C in a Beckman SW50.1 rotor to pellet the ribosomal subunits. Pellets were resuspended in 0.5 ml of gradient buffer and centrifuged in the microcentrifuge for 10 min to pellet insoluble matter, and then each subunit preparation was centrifuged again on a 0.5 to 1.0 M sucrose gradient. Fractions containing large and small subunits were pooled and extracted once with an equal volume of 1:1 phenol-chloroform and once with an equal volume of chloroform. RNA was precipitated from the final aqueous phase by addition of 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M and 3 volumes of ethanol. The quantity of RNA in each preparation was estimated by ethidium bromide fluorescence. The specific activity of label in each preparation was about 10^5 cpm/μg.

RNA binding assays. RNase T_1 protection binding assays were performed as previously described (27). The β-galactosidase/U_S11 fusion protein used for binding to rRNAs has been described previously (28). For binding reactions with fusion protein and rRNA preparations, 20,000 cpm of rRNA (about 200 ng) was reacted with 1 μg of fusion protein. The probe used for binding reactions with extracts of infected cells was a T7 transcript of *Pst*I-cut plasmid pRB3881. The structures of this plasmid and synthetic transcript have been previously described (27). The synthetic transcript contains sequences antisense to the 5′ transcribed, untranslated portion of the U_S11 mRNA and the U_S11 promoter.

Sucrose gradient fractionations. Cytoplasmic extracts were prepared from infected cell monolayers of 143B cells (5×10^7 cells) and fractionated on 0.5 to 1.0 M sucrose gradients as previously described (26).

Extraction of U_S11 protein from infected cells. Cell monolayers were washed twice in PBS, scraped into PBS, pelleted at 1,000 × *g* for 5 min, resuspended in 100 μl of 100 mM magnesium acetate–30 mM Tris (pH 7.4), and then solubilized and extracted by addition of 200 μl of glacial acetic acid. The extract was stored on ice for 30 min with occasional agitation to extract protein and to allow RNA and insoluble protein to precipitate. The precipitate was pelleted by centrifugation for 10 min in a microcentrifuge. The supernatant fluid was transferred to a fresh tube, and proteins were precipitated by adding 7 volumes of cold acetone. Precipitates formed during storage at −20°C for 1 h were pelleted by centrifugation for 5 min at 800 × *g* and washed by two cycles of resuspension and centrifugation in cold 90% acetone in water.

Samples for analysis by electrophoresis in denaturing gels were dissolved directly in disruption buffer containing sodium dodecyl sulfate (SDS) but without 2-mercaptoethanol, and the protein concentration was determined by using the Bio-Rad protein assay reagent.

Samples to be assayed for RNA binding activity were dissolved in 2 ml of U_S11 binding buffer (20 mM Tris [pH 7.5], 200 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 20% glycerol) containing 6 M guanidine hydrochloride. Proteins were renatured by six cycles of diafiltration through Centricon 10 ultrafiltration units (Amicon). In each cycle, samples were centrifuged to leave 0.7 ml of protein solution and then diluted to 2.0 ml with U_S11 binding buffer without guanidine. Following the final dilution, samples were concentrated to a volume of 100 μl. Extracts were stored at −80°C.

Purification of HSV-1 virions. Virions from wild-type and mutant HSV-1 were purified on dextran gradients by a modification of the method of Spear and Roizman (31) as previously described (28).

SDS-polyacrylamide gel electrophoresis and immunoblotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis, electrically transferred to a nitrocellulose sheet, and probed with antibody as previously described (28).

Immunofluorescence. Monolayer cultures of 143B cells grown on glass coverslips were maintained for 14 h after infection with 10 PFU of HSV-1(F) or of U_S11-mutant virus per cell, then washed with PBS, and fixed in 3.7% formaldehyde in PBS for 20 min at room temperature. After washing in PBS to remove fixative, the fixed cells were reacted with a 1:1,000 dilution of anti-U_S11 monoclonal antibody in IF buffer (PBS supplemented with 0.5% Triton X-100, 0.5% deoxycholate, and 1% bovine serum albumin). Coverslips were washed three times for 15 min each in PBS and then reacted with a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G in IF buffer containing 10% goat serum. Coverslips were then washed four times for 15 min each in PBS and mounted on glass slides in 95% glycerol, and cells were photographed under UV illumination.

Visualization of GFP. Monolayer cultures of 143B cells grown on glass coverslips were transfected with GFP constructs and then superinfected 24 h after transfection with 10 PFU of the mutant G U_S11 virus per cell. Fourteen hours after infection, coverslips were washed twice with PBS and cells were fixed by incubation in 3.7% formaldehyde for 20 min at room temperature. Coverslips were mounted on glass slides in 95% glycerol, and cells were photographed under UV illumination.

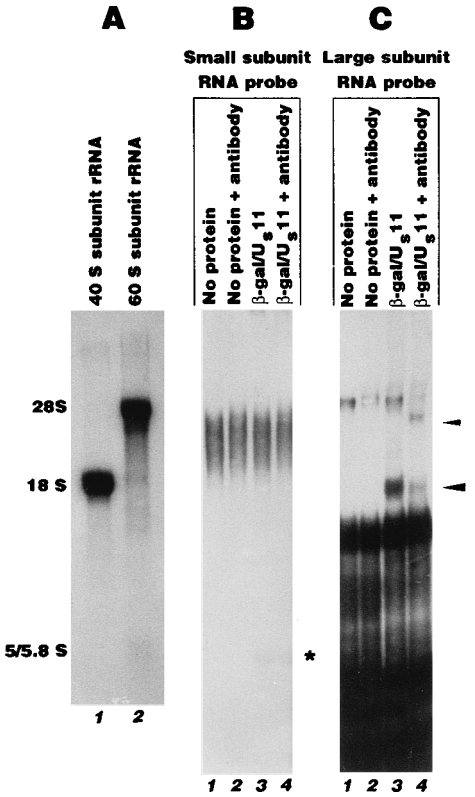

FIG. 2. Binding of β-galactosidase/U_S11 fusion protein to large subunit rRNA. (A) Autoradiographic image of metabolically labeled, purified RNA from small (lane 1) and large (lane 2) subunits of ribosomes. (B) Autoradiographic image of complexes formed between metabolically labeled small subunit rRNA depicted in panel A and either no protein (lane 1), anti-U_S11 monoclonal antibody alone (lane 2), β-galactosidase (β-gal)/U_S11 fusion protein (lane 3), and β-galactosidase/U_S11 fusion protein plus anti-U_S11 monoclonal antibody (lane 4). (C) Same as panel except that the RNA probe was large subunit rRNA.

RESULTS

Binding of U_S11 protein to rRNA. RNAs in uninfected HeLa cells were labeled with ^{32}P by growing cultures in the presence of $^{32}P_i$, and large and small ribosomal subunits were purified by repeated centrifugation on 0.5 to 1.0 M sucrose gradients. rRNA was purified from both subunit preparations by phenol-chloroform extraction, and their purity was assessed by agarose gel electrophoresis in the presence of formaldehyde (Fig. 2A). The RNA purified from small ribosomal subunits (lane 1) consists of a single species uncontaminated by large subunit RNA. The RNA purified from large subunits resolved into two major species, one large (28S rRNA) and one running near the dye front of the gel (5S rRNA and 5.8S rRNA). These RNAs were then used as probes in a binding-RNase T_1 protection assay with β-galactosidase/U_S11 fusion protein and anti-U_S11

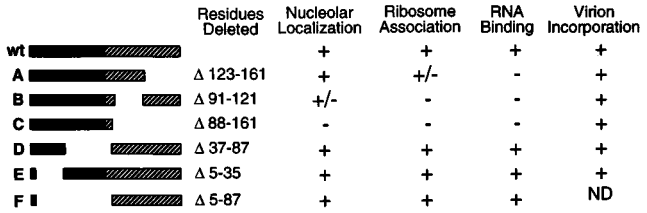

FIG. 3. Schematic depiction of the sequence arrangement in the mutant U_S11 proteins and their activities in RNA binding, association with ribosomes and ribosomal subunits, localization in nucleoli, and incorporation into virus particles. The nonrepetitive protein sequence in the N-terminal half of the protein is indicated by a filled bar. The three-residue RXP repeat comprising the C-terminal half of the protein is indicated by hatched bars. The amino acids deleted in each of the proteins are indicated to the right of each line. ND, not determined.

monoclonal antibody (Fig. 2B and C). RNAs were reacted either without (lanes 1 and 2) or with (lanes 3 and 4) 1 μg of β-galactosidase/U_S11 fusion protein followed by anti-U_S11 monoclonal antibody (lanes 2 and 4). Samples were then reacted first with RNase T_1 to degrade free RNA and RNAs bound nonspecifically and then with the polyanion heparin to further disrupt nonspecific RNA protein associations. Protected complexes were separated on a nondenaturing polyacrylamide gel. The lanes shown in Fig. 2B and C are from the same experiment and are shown at the same exposure. As is typical for this type of assay, each probe gives rise to a characteristic pattern of digestion products, some of which migrate quite slowly and are apparently quite large. These fragments presumably protect themselves from extensive digestion by adopting base-paired structures that cannot be cleaved by the single-strand-specific RNase T_1. In addition to its characteristic degradation pattern, labeled RNA isolated from purified large ribosomal subunits reproducibly gave rise to a protected complex (large arrowhead) that was dependent on the presence of the fusion protein and which could be supershifted by addition of anti-U_S11 monoclonal antibody. Labeled RNA isolated from purified small ribosomal subunits gave rise to no protected complexes that were dependent on the fusion protein and that could be supershifted with anti-U_S11 monoclonal antibody. The faint complexes marked with the asterisk were not present in all experiments and could not be supershifted with antibody. These results show that U_S11 protein can bind to one of the three large subunit rRNAs in vitro. While these results do not prove that U_S11 protein binds to 28S rRNA in vivo, they do suggest that the specificity of U_S11 association with ribosomes is reflected in the ability of U_S11 protein to bind to specific rRNAs in vitro.

Construction of U_S11 deletion mutant viruses. Viruses that express U_S11 genes carrying deletions were constructed in order to identify the amino acid sequences required for RNA binding and for ribosomal association. Mutants A and C were constructed by insertion of a linker containing a stop codon into the U_S11 protein coding sequence, resulting in the deletion of the carboxyl-terminal one-fourth and one-half of the U_S11 protein, respectively. Mutants B, D, E, and F were constructed by replacement of deleted restriction fragments by small linkers that maintain the reading frame. These mutations were first introduced into a plasmid-borne copy of the U_S11 gene and then recombined into the virus. The structures of the plasmids used for virus construction are summarized in Fig. 3. In addition, a novel U_S11 virus (mutant G) was constructed by insertion of a fragment containing the 3′ processing signal from the HSV-1 U_L26 gene into a *Bst*XI restriction site located downstream of the ICP47 termination codon and just upstream of the U_S11 initiation codon. This arrangement allows for expression of both α47 (with a 3′ truncated mRNA) and U_S10 genes while specifically ablating U_S11 gene expression. The structures of mutant A to G DNAs in the vicinity of the mutation were confirmed by Southern blotting (not shown).

To confirm that cells infected with the recombinant viruses accumulate U_S11 proteins with the expected structures, 143B cells were infected (10 PFU per cell) with either HSV-1(F) Δ305 or one of mutant viruses A through G for 18 h. Since wild-type U_S11 protein and all of the deletion mutants are rich in basic amino acid residues, especially arginine, they can be efficiently extracted from cells in 67% acetic acid. Infected cells were extracted with acetic acid, and the soluble fraction was precipitated with acetone. The precipitate was solubilized in disruption buffer containing SDS, subjected to electrophoresis on a denaturing 15% polyacrylamide gel, blotted to nitrocellulose, and probed with anti-U_S11 monoclonal antibody (Fig. 4A). The monoclonal antibody detected species of the expected apparent molecular weight in protein from cells infected with mutants A, B, C, and D, but no reactivity was observed with mutant E or F. We had expected that at least two of the mutants would fail to react with the monoclonal antibody because of deletion of the epitope sequences. Mutants E and F have in common the deletion of amino acid residues 5 to 35, suggesting that the epitope recognized by the monoclonal antibody consists at least partly of amino acid sequence in this region. To test for accumulation of the mutant proteins E and F and to further characterize the structure of the other mutants, a blot equivalent to that shown in Fig. 4A

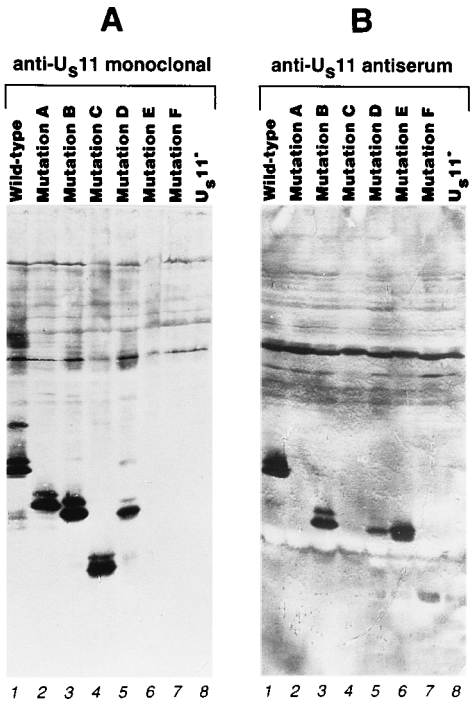

FIG. 4. Detection of wild-type and mutant U_S11 proteins in cells infected with recombinant viruses. (A) Photographic image of a blot of proteins from 143B cells infected with HSV-1 recombinants carrying either wild-type or mutant U_S11 genes, as indicated above the lanes, probed with anti-U_S11 monoclonal antibody. (B) Same as panel A but probed with antiserum raised against a carboxy-terminal peptide of U_S11 protein. The band comigrating with mutant protein F (seen most clearly in lanes 5, 6, and 8) was not observed in other experiments and is believed to be background resulting from overdevelopment necessary to visualize all of the mutants on the same blot.

was probed with an anti-U_S11 antiserum (gift of Howard Marsden) that was raised against a synthetic peptide corresponding to the carboxy-terminal 20 amino acids of U_S11 protein (10) (Fig. 4B). As expected, this antiserum failed to react with mutant proteins A and C but detected wild-type U_S11 protein and mutant proteins B, D, E, and F. The protein product of mutant F was detected only very weakly, and in all experiments this mutant protein accumulated much less efficiently than the other mutant forms of U_S11. It should be noted that in most experiments, mutant protein C also accumulated to significantly lower levels than the wild-type protein and the other mutant proteins.

Wild-type U_S11 migrates as a doublet in SDS-polyacrylamide gels. The nature of the modification responsible for this heterogeneity is not known, but U_S11 protein is known to be phosphorylated by cellular kinases (6, 30). An interesting feature of the results presented here is that all of the mutant forms of the U_S11 protein migrate as multiple species. The only sequences that all of the mutants have in common are the first four amino acid residues, suggesting either that these N-terminal four residues constitute the site of modification or that there are multiple modification sites on the protein and no one of them is uniquely responsible for the heterogeneous migration of wild-type U_S11 protein.

Sequences in the carboxy-terminal half of the U_S11 protein are both necessary and sufficient for association with the large subunit of ribosomes. To determine which domains of the U_S11 protein are required for association with the large subunit of ribosomes, cytoplasmic extracts of 143B cells that had been infected with wild-type and U_S11 mutant viruses were centrifuged on 0.5 to 1.0 M sucrose gradients. Gradient fractions were assayed for nucleic acid by A_{260} and for the presence of U_S11 protein by immunoblotting with either an anti-U_S11 monoclonal antibody (for wild-type and mutants A to D) or an anti-C-terminal peptide antiserum (for mutants E and F). The resulting immunoblots are shown in Fig. 5. The positions of the ribosomal subunit peaks are indicated above each blot. As we have demonstrated previously, only a small minority of the wild-type U_S11 protein in the extract is found at the top of the gradient with free protein. The vast majority of the wild-type protein cosediments with the 60S ribosomal subunits. U_S11 deletions B and C are found exclusively at the top of the gradients and show no detectable association with faster-migrating material. Though most of mutant protein A is found at the top of the gradient, a portion of it tails into the gradient to the position of the large ribosomal subunits. In contrast, mutants D and E sediment just like the wild-type protein. Testing the mutant F protein was problematic because it is accumulated in much lower amounts than the wild-type protein or other mutant proteins. In an initial experiment, cytoplasmic extracts from mutant F-infected 143B cells were fractionated on a sucrose gradient, and fractions were concentrated by precipitation with 60% ethanol and then resuspension in 10% of the original fraction volume. These gradient fractions were then analyzed by immunoblotting with the anti-U_S11 peptide antiserum. With this protocol, all of the immunoreactive material was found to cosediment with the large ribosomal subunits, indicating that mutant protein F can associate with ribosomes. We also observed, however, in a parallel experiment with wild-type U_S11 protein, that free U_S11 protein found at the top of the gradient is soluble under the conditions used for precipitation (not shown), suggesting that the pattern observed in Fig. 5 might not reflect the presence or distribution of any non-ribosome-associated mutant protein F. To address this possibility, cytoplasmic extract was prepared from 5×10^7 143B cells infected with mutant F for 18 h and centrifuged on a 0.5 to 1.0 M sucrose gradient. The gradient was fractionated, and fractions were analyzed by immunoblotting with anti-U_S11 peptide antiserum. Most of mutant protein F cosedimented in this experiment with the 60S subunits, but a small fraction was found at the top of the gradient (not shown).

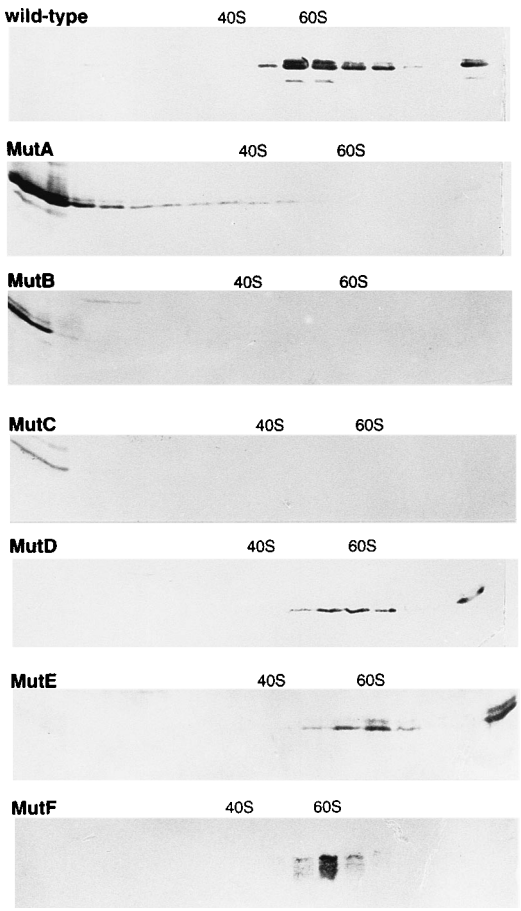

FIG. 5. Association of wild-type and mutant U_S11 proteins with the large subunit of ribosomes. Photographic images of blots of electrophoretically separated polypeptides from sucrose gradient fractionation of cytoplasmic extract of 143B cells infected with wild-type or U_S11-mutant virus, as indicated. Only the portion of the blot around the M_r of U_S11 protein (or mutant U_S11) is shown. The positions of the 40S and 60S ribosomal subunit peaks as determined from A_{260} are indicated above each panel.

Sequences in the carboxyl-terminal half of the U_S11 protein are necessary and sufficient for localization to nucleoli. The intracellular localization of U_S11 mutant proteins A to D was determined by immunofluorescence. Cultures of 143B cells infected with HSV-1(F)Δ305 or mutants A, B, C, D, and G for 14 h were fixed in formaldehyde and reacted for immunofluorescence studies with the anti-U_S11 monoclonal antibody as described in Materials and Methods. Results obtained with a conventional fluorescence microscope (Fig. 6) paralleled those seen with ribosomal association. Wild-type U_S11 protein (Fig. 6, panel 2) accumulated in both the cytoplasm and the nucleus, but was concentrated in the nucleoli. The fluorescence pattern in cells infected with mutant virus D (panel 6) could not be differentiated from that of cells wild-type virus. In contrast, fluorescent signal reflecting mutant B (panel 4) or C (panel 5) could not be detected in nucleoli. Cells infected with mutant virus A (panel 3) showed clear nucleolar staining, but less intense than that seen in wild-type or mutant D virus-infected cells.

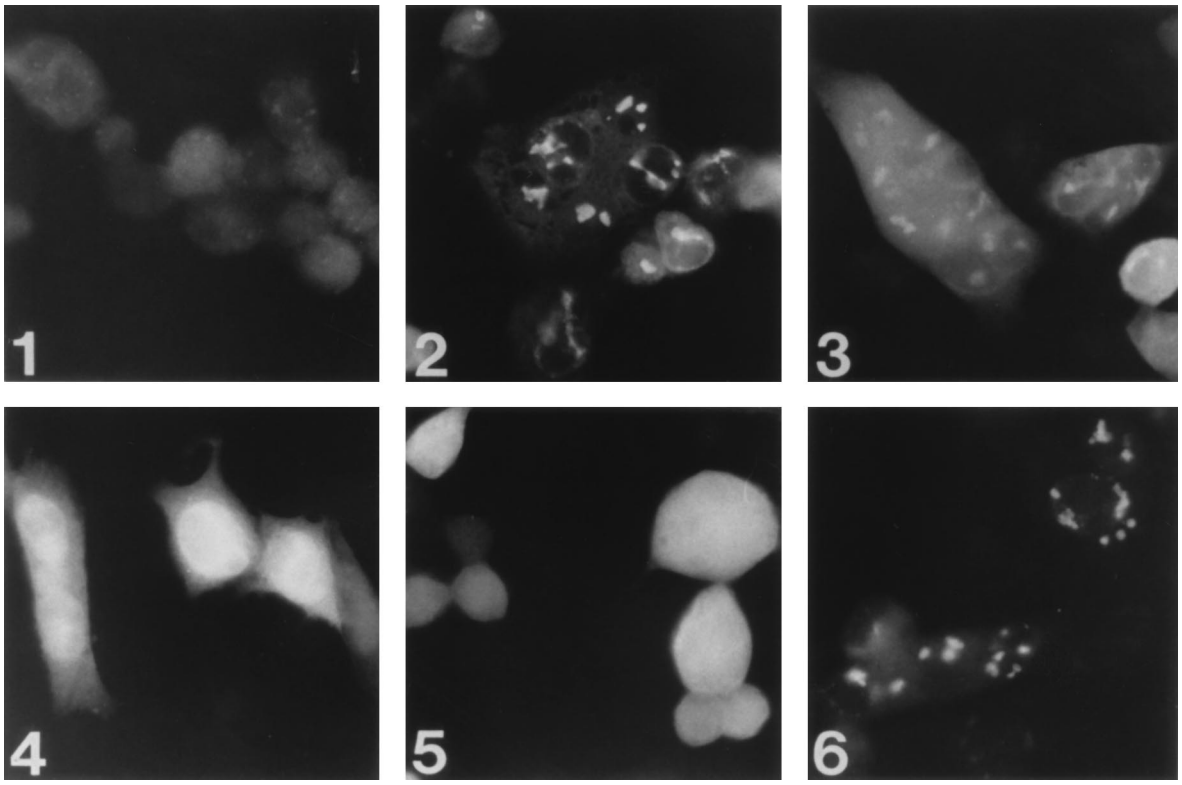

FIG. 6. Intracellular localization of wild-type and mutant U_S11 proteins. Photographic images of 143B cells infected with either mutant G U_S11^- virus (panel 1), HSV-1(F)Δ305 wild-type U_S11 virus (panel 2), mutant A (panel 3), mutant B (panel 4), mutant C (panel 5), or mutant D (panel 6), fixed, reacted with anti-U_S11 monoclonal antibody and fluorescein isothiocyanate-conjugated secondary antibody, and viewed with UV illumination.

These same immunofluorescent preparations were optically sectioned by confocal microscopy to look for nucleolar fluorescence that might be masked by the bright diffuse fluorescence observed with mutants B and C (Fig. 7). Again, wild-type U_S11 (panel 2) and mutants A and D (panels 3 and 6) were observed in nucleoli, and mutant C (panel 5) was not. In contrast to the results shown in Fig. 6, the mutant B protein (panel 4) could be detected in nucleoli.

Inasmuch as mutant proteins E and F lack the epitope for the anti-U_S11 monoclonal antibody, and since the anti-U_S11 antiserum gave unacceptable high backgrounds in immunofluorescence, we constructed plasmids in which the mutant E and F U_S11 proteins were fused to the carboxy-terminus of GFP. A control plasmid in which GFP alone is expressed under the control of the U_S11 promoter/regulatory sequences was also constructed (Fig. 1, lines 7, 9, and 11). Coverslip cultures of 143B cells were transfected with these plasmids and then superinfected with the U_S11^- mutant G virus. Fourteen hours after infection, cells were fixed, mounted, and photographed under UV light illumination in a conventional fluorescence microscope (Fig. 7, panels 7 to 9). When expressed by itself (panel 9), GFP shows a diffuse localization with no concentration in nucleoli. When fused to either the mutant E or F U_S11 sequence (panel 7 or 8, respectively), GFP fluorescence is concentrated in the cell nucleolus, demonstrating that the U_S11 sequences in these constructs can mediate localization to the nucleolus.

The failure of the mutant C U_S11 protein to localize to nucleoli shows that sequences in the carboxy-terminal half of the U_S11 protein are necessary for this activity of U_S11. The ability of the mutant F U_S11 sequences to direct localization of the mutant F-GFP fusion protein to the nucleoli shows that these same sequences are sufficient.

Sequences in the carboxy-terminal half of the U_S11 protein are necessary and sufficient for binding to specific RNA probe. To determine which amino acid sequences in the U_S11 protein sequence are required for RNA binding, RNA binding assays were done with extracts of cells infected with recombinant viruses and a synthetic RNA probe transcribed from pRB3881 (27). Wild-type U_S11 protein has been shown to bind to this probe in a site- and conformation-specific manner and to protect a portion of the probe from RNase T_1 digestion (27). Acetone precipitates of acetic acid extracts were prepared from 143B cells harvested 18 h after infection (10 PFU per cell) with HSV-1(F)Δ305 or with one of the mutant viruses A to G. Precipitated proteins were redissolved in buffer containing guanidine hydrochloride, renatured by diafiltration as described in Materials and Methods, and then reacted with probe. Reactions were digested with RNase T_1 to degrade free RNA and nonspecific RNA-protein complexes and then separated on a nondenaturing polyacrylamide gel (Fig. 8). Wild-type U_S11 protein from HSV-1(F)Δ305-infected cells formed the two protected complexes typically seen with this probe (lane 2) (27). Extracts from cells infected with mutant viruses A, B, and C failed to form any complexes, even though the mutant proteins were present in the extracts in amounts comparable to that of the wild-type protein (Fig. 8B; compare lanes 3 to 5 with lane 2). As usual, mutant C was present in lesser amounts than mutant A or B but was still present in sufficient amount to produce detectable complex if it bound as well as the wild type. In contrast, extracts from cells infected with mutants D, E, and F all formed complexes with the probe (Fig. 8A, lanes 7 to 9).

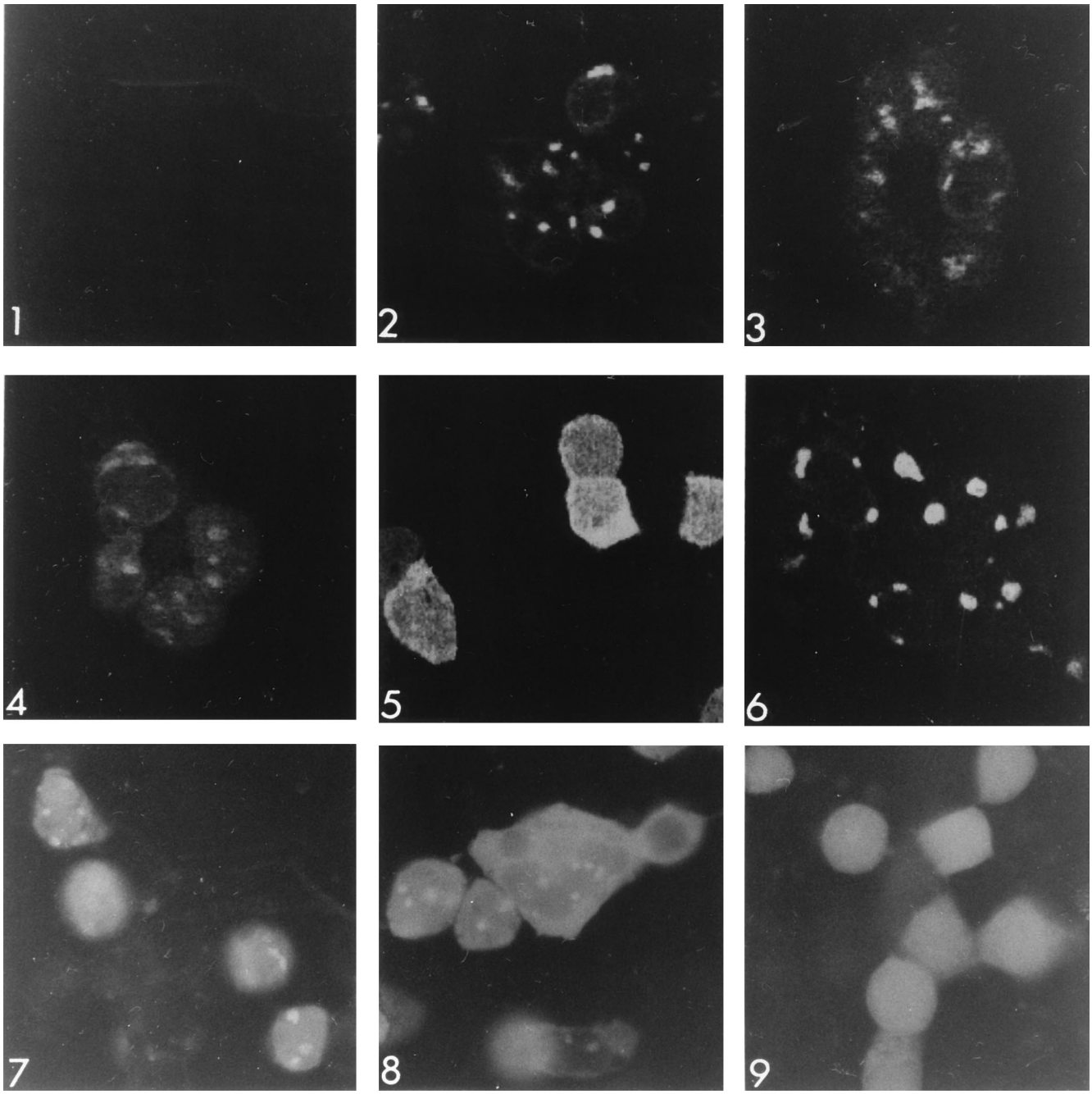

FIG. 7. Intracellular localization of wild-type and U_S11 mutant proteins and U_S11-GFP fusion proteins. Panels 1 to 6 show confocal images of 143B cells infected and treated as for Fig. 6. Panel 1, mutant G U_S11^- virus; panel 2, HSV-1(F)Δ305 wild-type U_S11 virus; panel 3, mutant A; panel 4, mutant B; panel 5, mutant C; panel 6, mutant D. Panels 7 to 9 show photographic images obtained with a conventional UV fluorescence microscope of 143B cells transfected with GFP plasmids, superinfected with mutant G U_S11^- virus, and fixed as described in Materials and Methods. Panel 7, pRR1029 mutation E-GFP fusion plasmid; panel 8, pRR1030 mutation F-GFP fusion plasmid; panel 9, pRR1031 mutation G-GFP plasmid. See Fig. 1 for the structures of the GFP plasmids.

These complexes all migrated more rapidly than the wild-type complex, presumably because of the smaller size of the protein component. The complexes formed by wild-type and mutant D U_S11 proteins could be supershifted with the anti-U_S11 monoclonal antibody (not shown). Attempts to supershift complexes formed by wild-type and mutant D, E, and F U_S11 proteins with the anti-U_S11 peptide antiserum were unsuccessful, suggesting that in the protein-RNA complex, the epitope of the immunogenic peptide was not accessible to the antibody.

Mutants A, B, and C are missing sequences from the carboxy-terminal half of the U_S11 protein, and this result suggests that these sequences are necessary for binding of U_S11 protein to RNA. Mutants D, E, and F have intact carboxy-terminal halves but are missing some (mutants D and E) or all (mutant F) of amino acids 5 to 87, suggesting that these deleted sequences are not necessary for RNA binding and that sequences from amino acid 88 to the carboxy terminus of the protein are both necessary and sufficient for RNA binding.

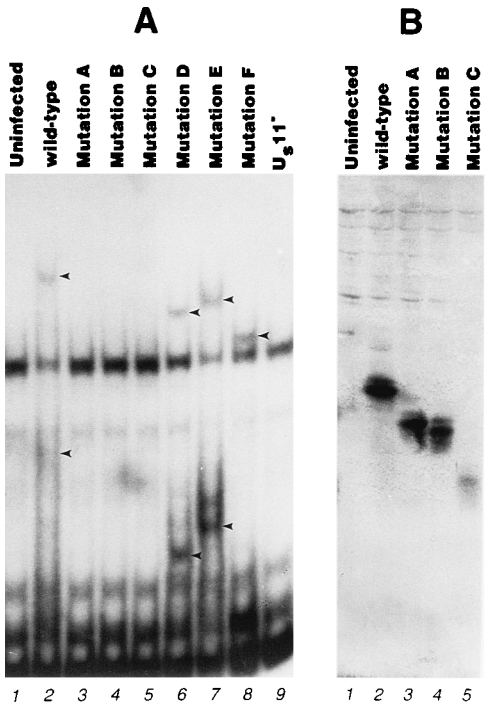

FIG. 8. Binding of wild-type and mutant U_S11 proteins to RNA. (A) Autoradiographic image of complexes formed between a pRB3881 T7 transcript probe and proteins from renatured acetic acid extracts of uninfected 143B cells and cells infected with wild-type or mutant virus, as indicated above the lanes. Arrowheads indicate the positions of specific RNA-protein complexes. (B) Photographic image of a blot of proteins from the same extracts used for the binding assay shown in panel A probed with anti-U_S11 monoclonal antibody.

Association of U_S11 mutants with HSV-1 virions. U_S11 protein is a component of the tegument of the HSV-1 virion, present in about 600 copies per virus particle (28). It seems highly likely that incorporation of U_S11 protein into the HSV-1 virion is the result of specific association of U_S11 protein with one or more other protein components of the virus particle. In an attempt to determine the sequences in U_S11 protein necessary for incorporation into virions, cultures of HEp-2 cells were infected with one of the U_S11 mutant viruses or with a virus [HSV-1(F)Δ305] expressing a wild-type U_S11 protein. Cytoplasmic extracts were prepared, and virus particles were purified on dextran gradients. Equal amounts of protein from each sample of purified virions were dissolved in disruption buffer containing SDS, subjected to electrophoresis on a denaturing polyacrylamide gel, transferred to nitrocellulose, and reacted with either anti-U_S11 monoclonal antibody for mutants A to D (Fig. 9A) or anti-U_S11 antipeptide antiserum for mutant E (Fig. 9B). Analysis of equivalent samples stained with Coomassie brilliant blue revealed that samples contained equivalent amounts of abundant virion proteins VP5 and VP16, suggesting that equivalent numbers of virions were loaded (not shown). Surprisingly, all of mutant proteins A to E were incorporated into virions, suggesting that no unique sequence is required for recruitment into the virion. Mutant protein C was incorporated at noticeably lower levels than the wild-type protein, possibly because of its lower accumulation during infection. Mutant F was not tested because of the low level of accumulation of mutant protein in the infected cell.

DISCUSSION

U_S11 protein binds specifically to RNA derived from the 60S ribosomal subunit. U_S11 protein in the form of a β-galactosidase/U_S11 fusion protein binds to and protects a fragment of RNA from the large ribosomal subunit but not the small subunit, suggesting that there is a specific binding site for the U_S11 protein in one of the three large subunit RNAs. Preliminary experiments have shown that when infected cell ribosomal subunits are digested with RNase T_1 and then immunoprecipitated with anti-U_S11 monoclonal antibody, multiple fragments of both 28S and 5.8S rRNAs are coprecipitated (28a). No fragments from the 5S rRNA have been observed in the immunoprecipitates, suggesting that the binding site(s) for U_S11 protein is located on 28S or 5.8S rRNA but not on 5S rRNA. The 28S and 5.8S rRNAs are bound together by intermolecular base pairs, and it is not yet clear whether U_S11 protein binds to only one or both of these RNAs.

The amino acid sequences required for binding of U_S11 protein to RNA and for association with ribosomes and nucleoli map in the carboxyl-terminal half of the protein. We constructed six mutant viruses that between them have deletions of all of the sequences of the U_S11 protein except the first four amino acids. The mutant forms of the U_S11 protein showed a good correlation between the ability to bind a specific RNA probe and the abilities to associate with the large subunit of ribosomes and to concentrate in nucleoli. Mutations with deletions in any or all of the N-terminal half of the protein were unimpaired in any of these three activities, whereas mutations in the carboxyl-terminal half of the protein show substantial impairments. The good correlation between RNA binding, ribosomal association, and nucleolar localization, together with the observation that U_S11 protein can bind to large subunit rRNA, suggests that U_S11 protein associates with the large subunit of ribosomes and concentrates in nucleoli by

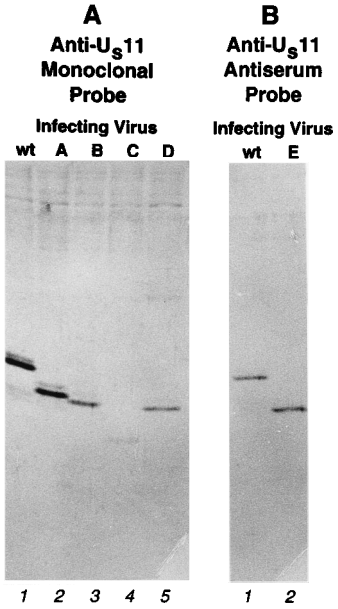

FIG. 9. Incorporation of wild-type and mutant U_S11 proteins into virions. Shown are photographic images of blots of electrophoretically separated proteins of virus particles purified from infected cell cultures. (A) Virion proteins from cells infected with wild-type (wt) virus (lane 1), mutant A (lane 2), mutant B (lane 3), mutant C (lane 4), or mutant D (lane 5) and probed with anti-U_S11 monoclonal antibody. (B) Virion proteins from cells infected with wild-type (wt) virus (lane 1) or mutant E (lane 2) and probed with anti-U_S11 antiserum.

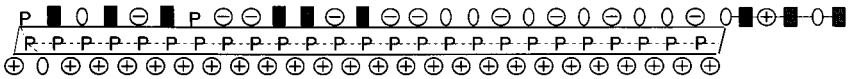

FIG. 10. Schematic depiction of proposed structure for the carboxy-terminal RNA-binding domain of U_S11 protein (residues 79 to 161). Arginine residues are indicated by circled plus signs. Proline residues are indicated with a P. Hydrophobic residues (Ile, Val, and Ala) are indicated with shaded rectangles. Polar residues (Ser, Thr, and Gln) are indicated with ovals. Acidic residues (Asp and Glu) are indicated by circled minus signs.

binding to a specific site on one or more of the large subunit rRNAs. The mutant A and B forms of U_S11 showed some distinctions in the ability to bind RNA and associate with ribosomes and nucleoli. Mutant protein A, missing the carboxyl-terminal 32 amino acids, showed a reduced but clearly detectable ability to associate with nucleoli but did not show detectable binding to the synthetic RNA probe in the assay shown in Fig. 8. During sucrose gradient fractionation, mutant protein A did not peak with the large ribosomal subunits as wild-type U_S11 protein does but rather tailed into the gradient, with a small fraction of the protein reaching the position of the large ribosomal subunits. This tailing during centrifugation could result either from dissociation of the protein from large ribosomal subunits during centrifugation or from formation of aggregates by the mutant protein. The observation that the mutant A protein shows a reduced association with nucleoli compared with the wild type suggests that mutant protein A may have a lower affinity for large ribosomal subunits and that the former explanation is correct. Mutant protein B, missing amino acids 91 to 121, showed greatly reduced but still detectable ability to localize in nucleoli but no detectable association with ribosomes during sucrose gradient fractionation or with specific RNA probe in the in vitro binding assay. We suspect that failure of mutant A and B U_S11 proteins to bind to the synthetic probe in vitro reflects a higher affinity of the U_S11 RNA-binding domain for its bona fide rRNA substrate than for the synthetic probe sequences. The association of both mutant proteins A and B with nucleoli suggests that sequences sufficient to direct localization may be redundant in the carboxy-terminal half of the protein, though the sequences present in mutant B serve this function only very poorly.

Diaz et al. have reported that under some conditions of extraction and fractionation, the association between U_S11 and large ribosomal subunits can be disrupted and that U_S11 distributes nonspecifically throughout a sucrose gradient (6). From these experiments, these authors have concluded that U_S11 makes no specific association with ribosomes. They also find that U_S11 protein may form extremely stable aggregates in dilute protein solutions and have suggested that the cosedimentation of U_S11 with ribosomes results from a combination of two effects: (i) nonspecific distribution of U_S11 throughout the sucrose gradient and (ii) detection of U_S11 protein only in the 60S region of the gradient as a result of the relatively high protein concentration in this region of the gradient. Several observations suggest that association of U_S11 protein with 60S ribosomal subunits is specific and likely to occur in the infected cell. (i) U_S11 fusion protein binds to rRNA in vitro, and this binding shows the same subunit specificity as is seen in association of the protein with intact ribosomal subunits in cytoplasmic extract. (ii) Specific mutations in the U_S11 protein sequence abolish the ability of U_S11 protein to associate with large ribosomal subunits. (iii) The association of U_S11 protein with nucleoli in the infected cell, as demonstrated by several methods (14, 23, 28), strongly suggests a specific affinity for some component of the ribosome. (iv) Mutations that abolish association with ribosomes also abolish or strongly inhibit the association with nucleoli, suggesting that they are reflections of the same phenomenon. Most of the infected cell U_S11 protein is tied up in associations with either ribosomal subunits or nucleoli, further suggesting that the function of the U_S11 protein is intimately tied to this association.

The U_S11 RNA-binding domain. The results shown in Fig. 8 demonstrate that a U_S11 mutant having only the carboxy-terminal sequences from residues 88 to 161 and five N-terminal amino acids can form a protective complex with RNA similar to that formed by the wild-type protein. Mutant forms of the protein missing part or all of these sequences cannot form such complexes. This result suggests that U_S11 protein has a discrete RNA-binding domain located in the carboxy-terminal half of the molecule and requiring for full activity sequences that are deleted in both mutant A and mutant B. The carboxyl-terminal RNA-binding domain of U_S11 protein does not contain any of the signature sequences that characterize many other RNA-binding proteins. The so-called RRM, KH, RGG, and Arg cluster sequences (reviewed in references 3 and 15) are not found anywhere in the U_S11 protein. Rather, the sequences responsible for U_S11 RNA binding and ribosomal association appear to represent a completely novel RNA-binding motif. As pointed out by Watson and Vande Woude (33) and Rixon and McGeoch (24), the carboxy-terminal half of the U_S11 protein consists of a tandemly repeated three-amino-acid motif with the sequence Arg-X-Pro, where X is frequently an acidic or uncharged polar amino acid residue (21 of 27 residues) and the number of repeats varies among HSV-1 strains. The regularity of sequence in this domain is highly suggestive of a corresponding regularity of structure. The presence of a proline residue at every third position suggests the formation of a poly-L-proline II helix secondary structure element by this sequence (1, 34). This structure is a left-handed single helix characterized by three residues per turn. Short regions of many globular proteins have been found to adopt this geometry (1, 32), and some pancreatic hormones and neuropeptides show extended regions with this conformation stabilized by a proline residue at every third position (2, 5). Williamson has proposed that extended poly-L-proline II helices are specialized for rapid and reversible binding to other proteins (34). There are as yet no other reports of RNA binding activity associated with this structure. Adoption of the poly-L-proline II helix by the U_S11 carboxyl-terminal domain has two functional implications. (i) As shown in Fig. 10, the predicted structure has a strong electrostatic polarity. One face of the helix is uncharged and relatively hydrophobic, because it exposes the ring atoms of the proline residues. A second face is highly positively charged, since it exposes the side chains of the repeating arginine residues. The third face exposes no positive charges but rather exposes a variety of hydrophobic, uncharged polar, and acidic side chains. Thus, two faces of the helix are uniform, and though the positively charged face may stabilize binding to RNA by electrostatic interactions with phosphates, specificity of binding must be provided by interactions of side chains from the heterogeneous face of the helix. (ii) Since poly-L-proline II helices are not stabilized by intrachain hydrogen bonds, they are thought to be relatively flexible rods, capable of fitting their conformation to that of a binding substrate.

Association of U_S11 protein with virions. U_S11 protein is a component of the HSV-1 virion and following infection is

released into the infected cell cytoplasm, where it associates with ribosomes. It seems likely that incorporation of U_S11 protein into the HSV-1 virion is mediated by interaction of some sequence of the U_S11 protein with another (probably protein) component of the virion. We observed that all of mutant proteins A to E are incorporated into virions. Though mutant protein C is incorporated into virions at lower levels than the wild-type protein or other mutant proteins, it is not clear if this is due to the lower accumulation of mutant protein C in infected cells or to a lower efficiency of incorporation. The key point is that there is no mutant U_S11 that completely fails to be incorporated into the virion. The only amino acid sequence common to all of these mutants is the N-terminal tetrapeptide, suggesting either that this peptide is responsible for virion incorporation or that the sequences responsible for interaction with the virion are redundant in the protein.

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