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# The Herpes Simplex Virus 1 RNA Binding Protein $U_S11$ Is a Virion Component and Associates with Ribosomal 60S Subunits

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**The herpes simplex virus 1 $U_S11$ gene encodes a site- and conformation-specific RNA binding regulatory protein. We fused the coding sequence of this protein with that of β-galactosidase, expressed the chimeric gene in *Escherichia coli*, and purified a fusion protein which binds RNA in the same way as the infected cell protein. The fusion protein was used to generate anti-$U_S11$ monoclonal antibody. Studies with this antibody showed that $U_S11$ protein is a viral structural protein estimated to be present in 600 to 1,000 copies per virion. The great majority of cytoplasmic $U_S11$ protein was found in association with the 60S subunit of infected cell ribosomes. $U_S11$ protein associates with ribosomes both late in infection at the time of its synthesis and at the time of infection after its introduction into the cytoplasm by the virion. $U_S11$ protein expressed in an uninfected cell line stably transfected with the $U_S11$ gene associates with ribosomal 60S subunits and localizes to nucleoli, suggesting that $U_S11$ protein requires no other viral functions for these associations.**

Herpes simplex virus 1 and 2 (HSV-1 and HSV-2) exhibit a tightly regulated pattern of gene expression for which the virus relies on a relatively large number of virally encoded regulatory proteins. We have previously reported on the regulatory properties of one such protein, the product of the HSV-1 $U_S11$ gene (32). This protein participates in a site- and conformation-specific RNA binding activity which requires no other viral factors (31). We have identified one viral RNA designated Δ34 as a substrate for $U_S11$ binding, and we have shown that its steady-state level is regulated by $U_S11$ such that it is six- to eightfold less abundant in cells infected with wild-type virus than in cells infected with a virus which does not express $U_S11$ (32). The Δ34 RNA is a truncated, possibly attenuated from of the $U_L34$ mRNA which encodes an essential protein (28), and which, like $U_S11$, is expressed late in infection.

The $U_S11$ open reading frame (22, 29, 40) encodes a polypeptide predicted to have an $M_r$ of ~18,000 and to be highly basic (29). $U_S11$ protein migrates in sodium dodecyl sulfate (SDS)-polyacrylamide gels as a doublet of $M_r$ ~21,000 (12). The migration varies between different strains of HSV, however, apparently due to variation in the number of the Arg-X-Pro repeats that compose the carboxyl-terminal half of the protein (29, 40). HSV-1 strain F [HSV-1(F)] produces a protein of approximately $M_r$ 23,000 (32). Neither the mechanism nor the significance of the regulatory activity of $U_S11$ have been determined, but two properties of the protein suggested avenues for investigation. (i) The $U_S11$ gene is expressed at high level and regulated as a $\gamma_2$ or true late gene (12). $U_S11$ gene product is thus a constituent of a group of proteins whose principal function is assembly and maturation of the virion. The high level of $U_S11$ protein expression and its synthesis as a late gene product are suggestive of a structural role for this protein. (ii) $U_S11$ protein is found in both the cytoplasm and nucleus of the infected cell, but nuclear $U_S11$ protein was reported to concentrate in the nucleolus (18). In this respect, in its size and charge, and in its possession of a site- and conformation-specific RNA binding and regulatory activity, the $U_S11$ protein is strikingly similar to the *tat* and *rev* proteins of human immunodeficiency virus (reviewed in reference 5). We have proposed that $U_S11$ protein may regulate transcription at the $U_L34/\Delta34$ locus by a mechanism analogous to that mediated by *tat* (32). The significance of the accumulation of Tat and Rev in the nucleolus is not yet clear, however. Unlike Tat and Rev, $U_S11$ protein is also present in the cytoplasm, and it seemed likely that its accumulation in the nucleolus might be explained and accompanied by a specific affinity of $U_S11$ protein for some component of ribosomes. With these issues in mind, we prepared a monoclonal antibody which we used to investigate the compartmentalization of the $U_S11$ protein in infected cells and in uninfected cells which express $U_S11$ protein constitutively.

Here we report that the $U_S11$ protein is a virion component. We also report that $U_S11$ protein associates with the 60S subunit of ribosomes during both the late phase of infection, when $U_S11$ protein is synthesized, and immediately after infection, at the time when it is introduced into the infected cell along with other components of the virion. The association with ribosomal 60S subunits can occur independently of other viral functions.

## MATERIALS AND METHODS

**Cells and viruses.** HSV-1(F) and the viral deletion mutant R3631 have been described elsewhere (6, 21). HEp-2 cells (M. A. Bioproducts) were propagated and infected as previously described (30). The construction and properties of the α4cl13 cell line have been described previously (1).

**Plasmid constructions.** Standard procedures were used in all constructions described in this report (19). The structures and details of construction of plasmids pRB4374, pRB4375, pRB4376, pRB4342, and pRB4354 are described in the text.

**Induction and purification of β-galactosidase–$U_S11$ fusion protein.** Exponentially growing cultures (optical density at 600 nm = 0.2) of *Escherichia coli* JM103 harboring plasmid pRB4376 were induced to synthesize fusion protein by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM and then shaken at 37°C for 6 h. Extraction of cells, ammonium sulfate fractionation of crude extract, and preparation of fusion protein for anion-exchange chromatogra-

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phy were done as described by Miller (23) for the purification of β-galactosidase. Protein was further fractionated by fast protein liquid chromatography (FPLC) on MonoQ resin (Pharmacia) with the buffers used by Miller (23) for DEAE-cellulose chromatography. Fractions were assayed for β-galactosidase activity (23), and those with the peak of activity were pooled, dialyzed against HAB (100 mM NaCl, 50 mM Tris [pH 7.5], 2 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol), and applied to a heparin agarose column. The heparin agarose column was washed with HAB and eluted with a linear gradient from 100 to 500 mM NaCl in HAB. Two peaks of β-galactosidase activity were recovered; one representing ~95% of the activity in the wash fractions, and another containing ~5% of the activity eluting at ~250 mM NaCl. Fractions from the latter peak were pooled and used for all experiments described here.

**Assays for RNA binding activity.** The generation and properties of a T7 RNA polymerase-transcribed, $U_S11$-binding probe from pRB3881 and methods for gel shift and RNase $T_1$ protection RNA binding assays were as previously described (31).

**Preparation of monoclonal antibody against $U_S11$ protein.** BALB/c mice, 8 to 10 weeks old, were infected by intraperitoneal injection of $10^6$ PFU of HSV-1(F) and then boosted 4 days later by intravenous injection of 50 μg of β-galactosidase–$U_S11$ fusion protein in phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$). Three days after boosting, spleen cells were harvested and fused as described previously (15) to NS-1 myeloma cells (13) and then cultured in medium containing hypoxanthine and thymidine (13). Culture supernatants from all viable colonies were screened by enzyme-linked immunosorbent assay (ELISA) both on unmodified β-galactosidase and on β-galactosidase–$U_S11$ fusion proteins. The culture supernatants which reacted with fusion protein but not with β-galactosidase were tested further on electrophoretically separated polypeptides from HEp-2 cells infected with HSV-1(F) or with R3631 ($U_S11^-$) and electrophoretically separated on denaturing polyacrylamide gels.

Two clones (28 and 109) reacted strongly and specifically with the fusion protein in ELISA and gave a strong reaction in immunoblot assay with a doublet of 23,000 $M_r$ only in HSV-1(F)-infected cells. Cells from these clones were used to generate ascites fluids. Ascites fluid from clone 28 was used for all experiments described in this report.

**Metabolic labeling of cells and immunoprecipitation.** Uninfected or HSV-infected HEp-2 cells were labeled by incubation of cell monolayers for 3 h in medium from which methionine was omitted and which was supplemented with 100 μCi of [$^{35}$S]methionine (>1,000 Ci/mmol; Amersham) per ml. Cell monolayers were then washed two times with PBS, scraped into PBS, and then pelleted by a 10-s centrifugation in an Eppendorf microcentrifuge at top speed. Cells were resuspended in extraction buffer (500 mM NaCl, 20 mM Tris [pH 7.5], 2 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 0.05% Triton X-100) and incubated with gentle mixing at 4°C for 30 min to extract nuclear protein. The nuclei were then pelleted by centrifugation for 10 min in a cold Eppendorf microcentrifuge at top speed. The supernatant fluid was adjusted to 1.0% Triton X-100, anti-$U_S11$ monoclonal ascites fluid was added to a final dilution of 1:400, and the mixture was reacted overnight at 4°C. A 0.1 volume of a 1:1 suspension of agarose-conjugated goat anti-mouse immunoglobulin G (Sigma) was added, and the reaction mixture was placed on a rotating wheel at 4°C for 1 h. The antibody-bound agarose was then pelleted by a 1-s spin in a microcentrifuge, washed three times in extraction buffer containing 1.0% Triton X-100 and 1 mg of bovine serum albumin per ml, and then washed once more with PBS. The immune complexes were solubilized by incubation in SDS-containing sample buffer at 65°C for 10 min.

**Purification of HSV-1 virions.** Virions were purified by a modification of the method described by Spear and Roizman (36). HEp-2 cells ($2 \times 10^8$ to $5 \times 10^8$) were infected at a multiplicity of 10 and incubated at 34°C for 48 h. To harvest, cell monolayers were washed twice with PBS, scraped into PBS, and then pelleted at $800 \times g_{max}$ in a graduated tube. The cell pellet was resuspended in 3.2 packed cell volumes of 1 mM $Na_2HPO_4$ (pH 7.4), transferred to a Dounce homogenizer, and placed on ice for 10 min to allow the cells to swell. Cells were broken with three strokes of the tight pestle, and nuclei were stabilized by the immediate addition of 0.8 packed-cell volume of 1.25 M sucrose and mixing with two more strokes of the pestle. The homogenate was then centrifuged at $15,000 \times g_{max}$ at 4°C for 10 min. The supernatant fluid was transferred to a Beckman SW50.1 polyallomer tube and centrifuged in an SW50.1 rotor at 30,000 rpm for 1 h at 2°C. The pellet, containing virions and other particulate matter, was resuspended by trituration in 0.5 ml of 1 mM $Na_2HPO_4$ (pH 7.4) and then thoroughly dispersed by sonication for 5 s with the microtip of a Branson Sonifier cell disruptor 200 at an output setting of 4. This suspension was layered on top of a 10-ml Dextran T10 (Pharmacia) gradient prepared as described by Spear and Roizman (36) in a Beckman SW41 polyallomer tube and then centrifuged in an SW41 rotor at 20,000 rpm for 1 h. The band of virions, which was found just above the middle of the tube, was harvested with a Pasteur pipette after all overlying material was aspirated. Urea was added to a concentration of 0.5 M, and the suspension was sonicated as described above. The volume of the suspension was then adjusted to 5.0 ml with 10 mM Tris (pH 7.4), and the suspension was centrifuged in an SW50.1 rotor at 30,000 rpm for 1 h at 2°C. The pellet, containing pure virions, was resuspended in a minimal volume of 10 mM Tris (pH 7.4).

**SDS-polyacrylamide gel electrophoresis (PAGE), staining, and immunoblotting.** Proteins were separated on SDS-polyacrylamide slab gels as described by Gibson and Roizman (9, 10). Gel staining with colloidal Coomassie blue was performed with the Pro-Blue staining system (Integrated Separation Systems), using the protocol supplied by the manufacturer. For immunoblotting, electrophoretically separated proteins were electrically blotted onto nitrocellulose and probed with anti-$U_S11$ monoclonal antibody as previously described (30).

**Sucrose gradient fractionations.** Cytoplasmic extracts of HEp-2 and $U_S11$cl19 cells were prepared and fractionated on 0.5 to 1.0 M sucrose gradients as previously described (32).

**Construction of the $U_S11$cl19 and $U_S11$cl19.1 cell lines.** α4cl13 cells were transfected with 10 μg of closed circular pRB4354 DNA by calcium phosphate coprecipitation and then glycerol shock (4). After 24 h, medium was replaced with medium containing 440 nM methotrexate. After selection for several weeks, individual cells were cloned by limiting dilution. One of these clones ($U_S11$cl19) was further subcloned by limiting dilution after several months of passage, when it was observed that considerable heterogeneity in expression of $U_S11$ protein had developed. The subclone expressing the highest amount of $U_S11$ ($U_S11$cl19.1), was used for immunofluorescence studies.

**Immunofluorescence.** Cell monolayers grown on 25-cm$^2$

tissue culture flasks (Corning) were washed twice with PBS and then fixed by adding ice-cold methanol and incubating at −20°C for 20 min. After fixation, methanol was removed by aspiration and the monolayer was allowed to dry for 1 h. Cells were rehydrated by a brief incubation in PBS and then exposed to a 1:1,000 dilution of anti-$U_S11$ monoclonal antibody in PBS for 2 h. Monolayers were washed three times for 10 min each in PBS and then exposed to a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Caltag Laboratories) for 1 h. After four 15-min washes in PBS, the bottom of the flask was cut out, a coverslip was applied over 95% glycerol in PBS, and cells were photographed under both phase-contrast optics and UV illumination.

## RESULTS

**Preparation of a $U_S11$–β-galactosidase fusion protein.** Initial attempts to express $U_S11$-coding sequences as a component of a fusion protein in *E. coli* by using several different fusion partners and host strains were unsuccessful, because of the instability of the $U_S11$ moiety of the fusion proteins. The product of such attempts was usually a protein of a size which could not be differentiated from that of the unmodified fusion partner. We therefore attempted to produce a fusion protein with a modified form of $U_S11$. We have previously shown that $U_S11$ protein can accept small additions to its carboxyl terminus without effect on its specific RNA binding activity (31). The $U_S11$ protein-coding sequence was modified by mutagenesis to introduce an *Xba*I site at the position of the termination codon (Fig. 1B), and this site was used to introduce a 20-amino-acid extension (Fig. 1C). This tagged $U_S11$ sequence was then cloned into the fusion vector pUR288 (33) at the 3′ end of the β-galactosidase protein-coding sequence (Fig. 1D). Once again, the major product observed upon induction with IPTG was a species of $M_r$ 116,000, i.e., indistinguishable from the unmodified β-galactosidase. However, a small but detectable amount of a larger species having the $M_r$ (140,000) predicted for the full-length fusion protein was also produced. Both species were purified together by ammonium sulfate fractionation and MonoQ FPLC. The full-length fusion protein was then enriched by chromatography on heparin agarose, to which $U_S11$ and the β-galactosidase–$U_S11$ fusion protein bind, but unmodified β-galactosidase does not. A Coomassie blue-stained SDS-PAGE profile of the heparin agarose-purified material is shown in Fig. 2A. Protein of $M_r$ 116,000 is still the major species, possibly because of the formation of mixed tetramers containing three subunits of $M_r$ 116,000 and one of $M_r$ 140,000.

**β-galactosidase–$U_S11$ fusion protein binds RNA.** Heparin agarose-purified fusion protein was tested for RNA binding activity in both gel shift and RNase $T_1$ protection assays (Fig. 2B). For gel shift assay, uniformly labeled in vitro transcripts of pRB3881 which have been shown to contain a $U_S11$ binding site (29) were reacted with either no protein (lane 1) or with 100 ng of protein (lane 2) from the heparin agarose pool shown in Fig. 2A and then electrophoretically separated on a nondenaturing polyacrylamide gel. As usual, free probe (Fig. 2B, lane 1) ran as two major species which are conformational isomers (31). Reaction with the heparin agarose pool resulted in the formation of a slower-migrating complex (arrowhead), indicating the presence of RNA binding activity. For the RNase $T_1$ protection assay, probe and protein were reacted in the same way as for the gel shift assay, except reactions were digested with RNase $T_1$ just before electrophoresis. A complex of protected RNA probe and protein (arrowhead) was formed in the presence of fusion protein (Fig. 2B, lane 4), but not in its absence (lane 3), indicating the presence of an RNA binding and protecting

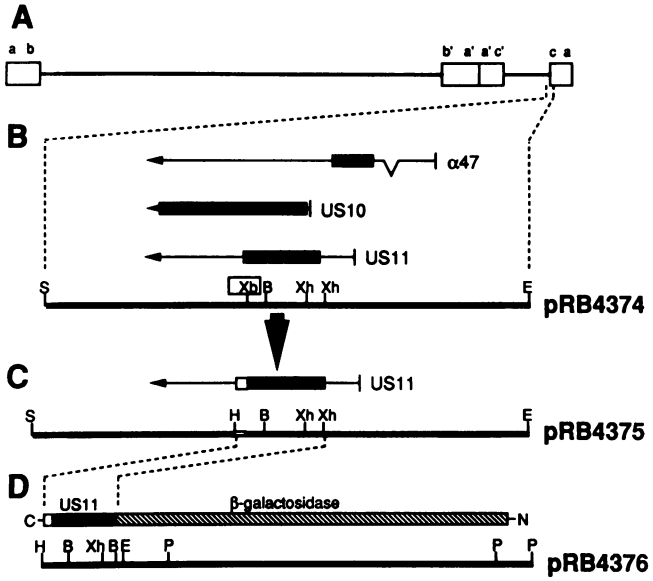


FIG. 1. Sequence arrangement of the HSV-1 genome and of the region around the $U_S11$ gene, and construction of pRB4376, encoding a β-galactosidase–$U_S11$ fusion protein. (A) Schematic diagram of the HSV-1 genome in the prototype arrangement, showing the unique sequences (lines) flanked by inverted repeats ab,b′a′a′c′, and ca (rectangles); (B to D) expansion of the region of the S component of the genome cloned in pRB4374 and showing the arrangement of the α47, $U_S11$, and $U_S10$ genes contained within these sequences and construction of pRB4376. (B) *Eco*RI-*Sal*I fragment of pRB421 (21) was cloned into pGEM-3Z(f+) (Promega Biotec) and subjected to site-directed mutagenesis to introduce a unique *Xba*I site at the position of the $U_S11$ termination codon to generate pRB4374, changing the sequence from GGG TCT GTA TAG CCC GGG CAA (termination codon underlined) to GGG TCT GTt cta gaC GGG CAA (mutant nucleotides in lower case). (C) pRB4374 was opened at the unique *Xba*I site, and a double-stranded oligonucleotide with *Xba*-compatible ends was inserted to generate pRB4375. The inserted oligonucleotide had the sequence (nontemplate strand) 5′-CTA GGC AAA GGC CAG AAA CCC AAC CTG CTG GAT AGC CTG AGC CAC AGC AAA AAC GGC TAT AGCV CAC TAA GCT-3′ (the template strand was of complementary sequence, except that on both strands there was a non-base-paired 5′-CTAG overhand). This sequence extended the $U_S11$ open reading frame by 20 amino acids, the extension having the sequence LGKGQKPN LLDSLSHSKNGYSH-term. The insertion of the oligonucleotide also introduced a *Hin*dIII site at the termination codon of the extended open reading frame. (D) Complete extended $U_S11$ protein-coding sequence was excised from pRB4375 by complete digestion with *Hin*dIII and partial digestion with *Xho*I and was ligated into *Sal*I-*Hin*dIII-cut pUR288 to generate pRB4376. Only the portion of pRB4376 between the *Pvu*II site just 5′ to the β-galactosidase promoter and the *Hin*dIII site at the 3′ end of the $U_S11$ protein-coding sequence is shown. Vertical bars indicate transcription initiation sites; horizontal lines indicate transcribed noncoding domains; solid boxes indicate HSV-1 protein-coding sequences; open boxes indicate tag protein-coding sequences; partially filled box indicates β-galactosidase protein-coding sequence. Restriction enzyme abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; S, *Sal*I; Xb, *Xba*I; Xh, *Xho*I. The boxed Xb indicates the position of an *Xba*I site introduced at the position of the $U_S11$ termination codon by site-directed mutagenesis.

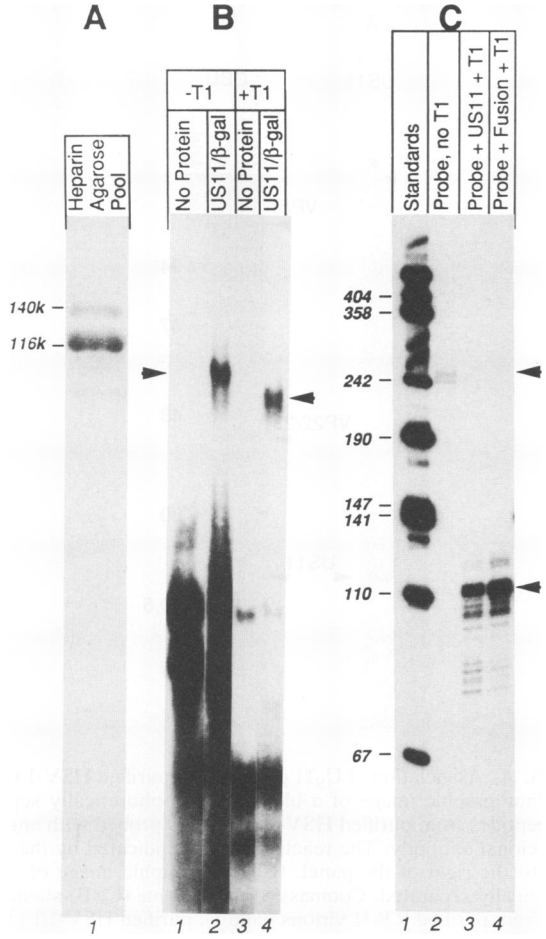


FIG. 2. Composition and binding activity of the purified β-galactosidase–$U_S11$ fusion protein and specificity of monoclonal antibody derived with fusion protein antigen. (A) Photographic image of polypeptides from a preparation of fusion protein electrophoretically separated in a denaturing gel and stained with Coomassie blue. The positions and $M_r$s of the two major species are indicated to the left of the panel. k, $\times 10^3$. (B) Autoradiographic images of electrophoretic separations of complexes formed between pRB3881 T7 transcript probe and no protein (lanes 1 and 3) and 100 ng of purified fusion protein (lanes 2 and 4). Samples in lanes 1 and 2 were not digested with RNase before electrophoresis. Samples in lanes 3 and 4 were digested with 10 U of RNase $T_1$ before electrophoresis. The positions of specific shifted and protected complexes are indicated by arrowheads to the left and right of the panel, respectively. (C) Autoradiographic images of denaturing gel electrophoresis of RNA purified from RNase $T_1$-digested RNA-protein complexes. Lane 1, *Msp*I-digested, end-labeled pGEM-3Z molecular weight standards (fragment lengths shown to the left of the panel); lane 2, undigested pRB3881 T7 transcript probe RNA; lane 3, protected RNA from RNase $T_1$-digested HSV-1(F) infected cell complex; lane 4, protected RNA from RNase $T_1$-digested fusion protein complex.

activity. To show that the probe and fusion protein form a complex like that formed by probe and $U_S11$ from HSV-1-infected cells, we determined the sizes of the RNA fragments protected from RNase $T_1$ digestion by infected cell and fusion protein complexes. RNA probe was reacted with HSV-1(F)-infected cell extract or with fusion protein, digested with RNase $T_1$, and electrophoretically separated on a nondenaturing gel. Binding complex bands, like that seen in Fig. 2B, lane 4, 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 size standards (Fig. 2C). The RNA fragments protected by infected cell extract (Fig. 2C, lane 3) and by fusion protein (lane 4) showed identical patterns, indicating that complexed RNAs are protected in the same way.

**Preparation and specificity of anti-$U_S11$ monoclonal antibody.** Monoclonal antibody to $U_S11$ was prepared as described in Materials and Methods, using β-galactosidase–$U_S11$ fusion protein as the antigen for immunization of mice. The specificity of the antibody produced by clone 28, used for experiments in this study, was evaluated by immunoprecipitation. HEp-2 cells were infected at a multiplicity of 5 with either HSV-1(F) or the deletion mutant R3631, which does not express $U_S11$ protein (21). After 16 h of infection, the cells were incubated in medium containing 67 μCi of [$^{35}$S]methionine per ml for an additional 3 h. Cellular extracts were prepared and incubated sequentially with anti-$U_S11$ monoclonal clone 28 and agarose-conjugated secondary antibody. The immune complexes were washed, eluted, and subjected to electrophoresis in denaturing polyacrylamide gels (Fig. 3B), along with a portion of the extracts from labeled cells (Fig. 3A). A doublet of $M_r$ ~23,000 characteristic of $U_S11$ protein was immunoprecipitated from cells infected with wild-type virus (Fig. 3B, lane 2), but not from cells infected with R3631 (Fig. 3B, lane 1).

**$U_S11$ protein is a component of the virion.** $U_S11$ protein is the product of a viral $\gamma_2$ gene (12). Other such genes whose functions have been characterized either are components of the mature virion or are otherwise involved in the assembly and maturation of virions. Two types of experiments were done to demonstrate the presence of $U_S11$ protein in the HSV-1 virion. (i) Anti-$U_S11$ monoclonal antibody was used to probe nitrocellulose blots of electrophoretically separated proteins of virions purified from cultures of HSV-1(F)-infected HEp-2 cells (Fig. 4A). A strong signal with the $M_r$-23,000 and doublet appearance characteristic of $U_S11$ protein was detected (Fig. 4A, arrowhead). (ii) Virions were purified from HEp-2 cells infected with either HSV-1(F) or the $U_S11^-$ virus R3631, subjected to electrophoresis in denaturing gels, and stained with Coomassie brilliant blue (Fig. 4B). A band with the same $M_r$ as $U_S11$ was detected among the proteins from wild-type virions (Fig. 4B, lane 2, arrowhead). This band contained the $U_S11$ protein and not another comigrating species inasmuch as it was not present among electrophoretically separated proteins of virions purified from cells infected with R3631 (Fig. 4B, lane 1). The relative amount of $U_S11$ in virions was determined on the basis of the calculations of Heine et al. (11) from the $U_S11$ peak area obtained by densitometry of Fig. 4B, lane 2, compared with that of VP5 and combined VP22/23, whose amount in the virion has been previously determined (11). These measurements indicate that $U_S11$ protein present in virions constitutes ~8% of the mass of VP5 and ~14% of the mass of VP22/23. These percentages correspond to estimates of about 600 and 1,000 molecules of $U_S11$ per virion, respectively. Thus, $U_S11$ protein is a relatively minor component of the virion by mass, but a major component in number of molecules per virion.

**$U_S11$ protein associates with the 60S subunit of the ribosome.** In earlier studies, the $U_S11$ protein was found in both the cytoplasm and nucleus of the infected cell and has been reported to localize in the nucleoli of infected BHK cells (12). The following experiments were done to test the hypothesis that this localization is due to affinity for a specific component of the ribosome. Cytoplasmic extracts

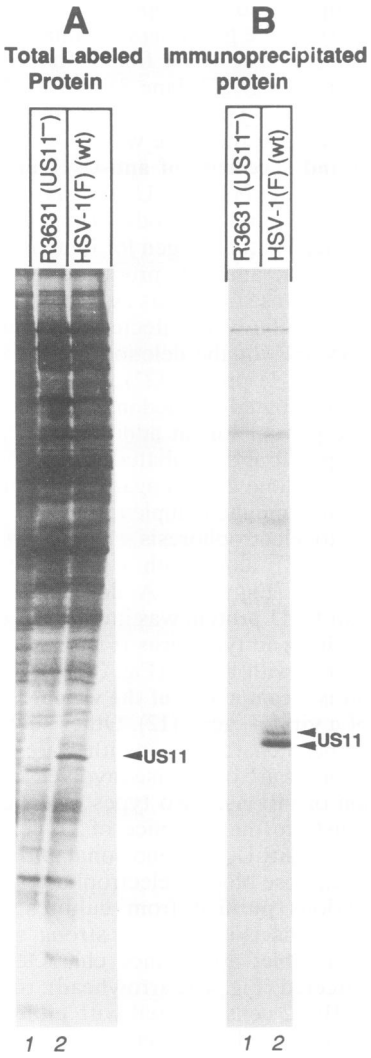


FIG. 3. Immunoprecipitation of $U_S11$ protein by anti-$U_S11$ monoclonal antibody. Autoradiographic images of [$^{35}$S]methionine-labeled, electrophoretically separated polypeptides. (A) Total extracts from cells infected with R3631 (lane 1) and HSV-1(F) (lane 2). (B) Protein immunoprecipitated from extracts shown in panel A. Lane 1, R3631; lane 2, HSV-1(F).

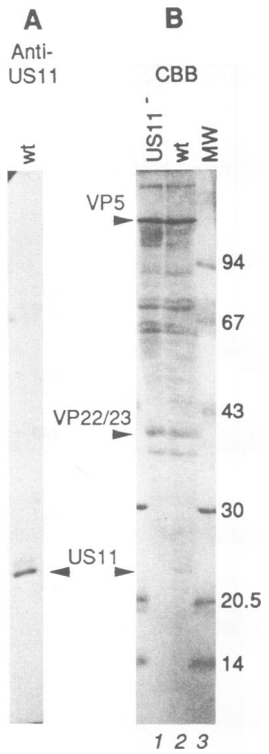


FIG. 4. Association of $U_S11$ protein with purified HSV-1 virions. (A) Photographic image of a blot of electrophoretically separated polypeptides from purified HSV-1(F) virions probed with anti-$U_S11$ monoclonal antibody. The reactive band is indicated by the arrowhead to the right of the panel. (B) Photographic image of electrophoretically separated, Coomassie brilliant blue (CBB)-stained proteins from purified R3631 virions (lane 1), purified HSV-1(F) virions (lane 2), (wt, wild type), and size standards (lane 3) (mw, molecular weight). The migration positions of $U_S11$ protein and of those proteins (VP5 and VP22/23) used for estimation of the amount of $U_S11$ protein are indicated by arrowheads at the left of the panel. The $M_r$s ($\times 10^3$) of the size standards used are indicated to the right of the panel.

prepared from HEp-2 cells infected with 10 PFU of HSV-1(F) per cell at 18 h after infection were centrifuged on 0.5 to 1.0 M sucrose gradients, and gradient fractions were assayed for both nucleic acid (by $A_{260}$) and $U_S11$ protein (Fig. 5). The absorbance pattern obtained (Fig. 5A) was typical of HEp-2 cells at this phase of infection, in which most ribosomal subunits were dissociated and only a minor fraction were engaged in protein synthesis as either monosomes or polysomes. The vast majority of $U_S11$ protein cosedimented with the 60S large subunit of ribosomes. Only a very minor fraction remained at the top of the gradient with most of the nucleic acid, and no $U_S11$ protein was detectable in fractions containing 40S subunits, suggesting that cosedimentation of $U_S11$ and 60S subunits is due to a specific interaction and not to a nonspecific affinity of $U_S11$ protein for RNA. Some $U_S11$ protein also colocalized with more rapidly sedimenting structures that contain 60S subunits such as 80S monosomes and was also present in the polysomal pellet, suggesting that $U_S11$ protein can associate with fully formed ribosomes.

**Virion-borne $U_S11$ protein associates with the 60S subunit of the ribosome.** Since $U_S11$ protein is a component of the virion, it may be introduced into the host cell along with other virion components at the time of infection. To determine whether virion-borne $U_S11$ protein also associated with ribosomal components, we infected HEp-2 cells at high multiplicity (100 PFU per cell) in the presence of phosphonoacetic acid to prevent viral DNA replication and subsequent de novo $U_S11$ expression, and a cytoplasmic extract was prepared at 2 h after infection. This extract was centrifuged on a 0.5 to 1.0 M sucrose gradient, and gradient fractions were assayed both for nucleic acid (by $A_{260}$) and for $U_S11$ protein (Fig. 6). Just as with $U_S11$ protein synthesized at late times, the great majority of $U_S11$ protein brought in by the virion was found to cosediment with the 60S ribosomal subunits and faster-sedimenting species.

**$U_S11$ protein associates with ribosomal components independent of other viral functions.** $U_S11$ protein may associate with nucleoli and the 60S component of ribosomes either because of its own affinity for ribosomal constituents or because it is associated with some other viral factor that has

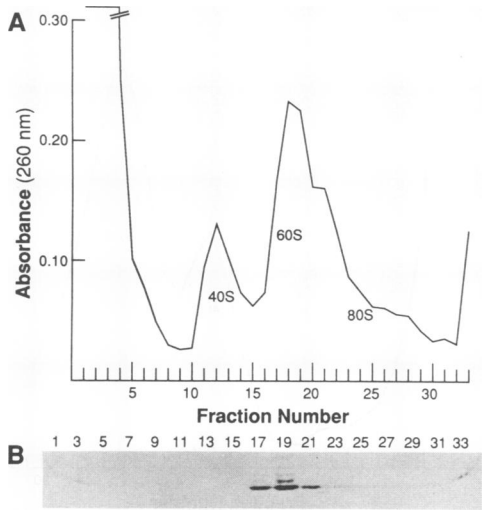


FIG. 5. Association of $U_S11$ protein with the 60S subunit of ribosomes. (A) Graph of $A_{260}$ versus fraction number for sucrose density gradient fractionation of cytoplasmic extract from ~5 × $10^7$ HEp-2 cells infected at a multiplicity of 10 for 18 h. Sedimentation is from left to right. One-third of each fraction was used for determination of absorbance. Designations of absorbance peaks as 40S, 60S, and 80S are based on gel electrophoretic analysis of RNA isolated from fractions of a similar gradient (e.g., the designation of 60S indicates the peak of 28S rRNA). (B) Photographic image of SDS-PAGE and immunoblot analysis of odd-numbered fractions from the gradient shown in panel A. Only the portion of the blot around the $M_r$ of $U_S11$ protein is shown. One-tenth of each odd-numbered fraction was used for assay.

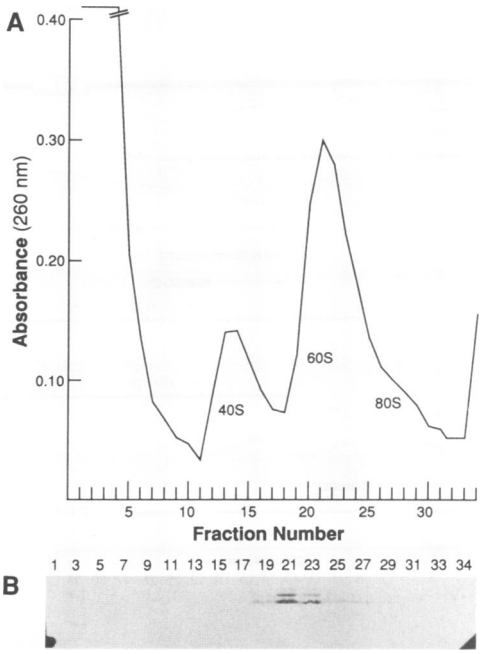


FIG. 6. Association of virion-borne $U_S11$ protein with the 60S subunit of ribosomes. (A) Graph of $A_{260}$ versus fraction number for sucrose density gradient fractionation of cytoplasmic extract from ~5 × $10^7$ HEp-2 cells infected at a multiplicity of 100 with HSV-1(F) for 2 h. Sedimentation is from left to right. One-third of each fraction was used for determination of absorbance. (B) Photographic image of a blot of electrophoretically separated polypeptides from odd-numbered fractions (and including the pellet fraction, 34) of the gradient depicted in panel A, probed with anti-$U_S11$ monoclonal antibody. Only the portion of the blot around the $M_r$ of $U_S11$ protein is shown.

affinity for the 60S ribosomal subunit. To differentiate between these alternatives, we determined the subcellular location of $U_S11$ protein in a cell line that expresses the $U_S11$ gene constitutively. To this end, the $U_S11$ protein-coding sequence and polyadenylation signal under the control of the HSV-1 gB promoter were cloned into pFR400 (35) (Fig. 7), this plasmid was transfected into α4cl13 cells (1), and stable transformants were selected by incubation in the presence of methotrexate. Previous work from this laboratory (1) has shown that the gB promoter can drive gene expression at a high level and in an α4-dependent manner in α4cl13 cells, which express α4 protein constitutively. Methotrexate-resistant cells were cloned by limiting dilution and tested for $U_S11$ expression in an immunoblot assay (Fig. 7F). Clones with a wide range of expression levels were obtained, and the one which expressed the highest level of $U_S11$ protein, designated $U_S11$cl19, was selected for further study. To determine whether $U_S11$ protein produced in $U_S11$cl19 cells associated with the 60S subunit of ribosomes, we prepared cytoplasmic extract from cells that had been incubated in medium containing 250 μM puromycin for 30 min to induce disaggregation of polysomes, and the extract was centrifuged on a 0.5 to 1.0 M sucrose gradient. Gradient fractions were assayed for nucleic acid (by $A_{260}$) (Fig. 8A) and for the presence of $U_S11$ protein (Fig. 8B). Just as in HSV-infected cells, most of the cytoplasmic $U_S11$ protein cosedimented with the 60S ribosomal subunit. The results of this experiment indicate that no viral factor other than ICP4, which was required for the expression of the $U_S11$ gene, was necessary for the association of $U_S11$ protein with ribosomes.

To determine whether $U_S11$ protein also concentrates in nucleoli in this cell line, monolayer cultures of $U_S11$cl19.1 (Fig. 9A and B) and α4cl13 cells (Fig. 9C and D) were fixed and incubated with anti-$U_S11$ monoclonal antibody and stained with fluorescein isothiocyanate-conjugated secondary antibody. These studies showed that nucleoli of $U_S11$cl19.1 cells exhibited specific fluorescence due to $U_S11$ (Fig. 9B).

## DISCUSSION

The results of experiments presented in this and prior reports show that $U_S11$ protein displays an astonishingly complex range of activities and that for most of these, $U_S11$ protein requires no other viral functions.

**RNA binding activity of β-galactosidase–$U_S11$ fusion protein.** We showed here that a purified β-galactosidase–$U_S11$ fusion protein binds to a specific RNA probe in the same way as the authentic $U_S11$ protein from extracts of HSV-1-infected cells. This result is of interest from two points of view.

(i) $U_S11$ synthesized in HSV-1-infected cells has a site- and conformation-specific RNA binding activity (31). $U_S11$ protein requires no other viral functions to express this activity inasmuch as the activity can be generated in rabbit reticulocyte lysate upon translation of a synthetic $U_S11$ mRNA (31). The binding of the purified fusion protein made in bacteria demonstrates that no eukaryotic accessory factors or modifications of $U_S11$ protein are required for binding. This result further suggests that $U_S11$ protein is completely sufficient for specific RNA binding. Proof of this

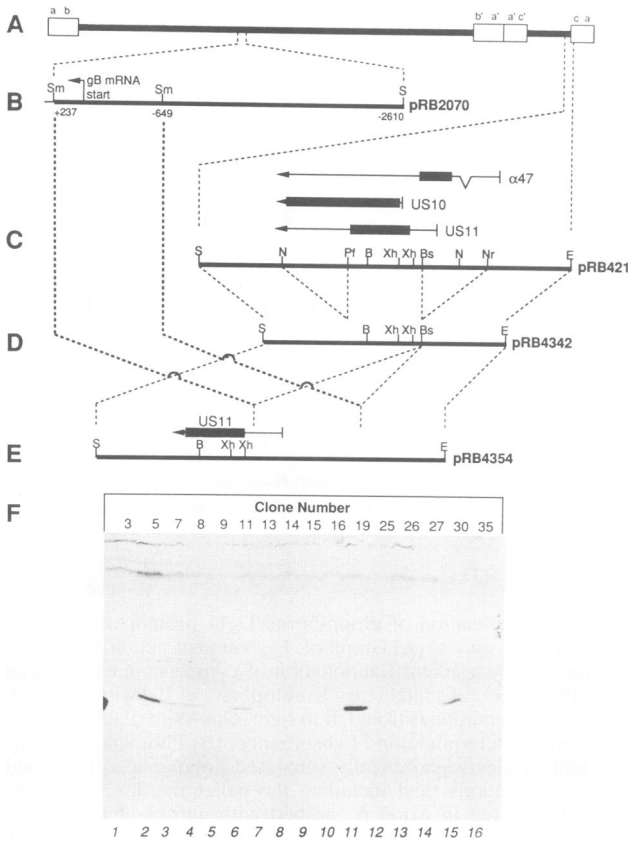


FIG. 7. Construction of a $U_S11$-expressing cell line. Restriction site abbreviations used: B, *Bam*HI; BS, *Bst*EII; E, *Eco*RI; N, *Eco*NI; Nr, *Nru*I; Pf, *Pfl*MI; S, *Sal*I; Sm, *Sma*I; Xh, *Xho*I. Panels A to C show the location and arrangement of HSV-1 sequences used for cell line construction. (A) Schematic diagram of the HSV-1 genome in prototype arrangement showing the unique sequences (filled bars) flanked by inverted repeats (open boxes). (B) Expansion of the region of the HSV-1 genome cloned in pRB2070 (26), showing the positions of the gB mRNA transcription initiation site (bent arrow) and of the restriction sites used for excision of the gB promoter fragment. Numbers underneath the line indicate nucleotide position with respect to the transcription initiation site of gB mRNA. (C) Expansion of the region of the HSV-1 genome cloned in pRB 421 (21). Vertical bars indicate transcription initiation sites; thin lines indicate transcribed noncoding domains; solid boxes indicate open reading frames α47, $U_S11$, and $U_S10$. Panels D to E show the construction of a gB promoter-driven $U_S11$ gene. (D) pRB421 was digested with *Bst*EII and *Nru*I, treated with Klenow fragment in the presence of deoxynucleotide triphosphates (dNTPs), and then religated to generate pRB4028 (not shown). pRB4028 was digested with *Pfl*MI and *Eco*NI, treated with T4 DNA polymerase, and religated to generate pRB4342. (E) The 886-bp *Sma*I fragment from pRB2070 containing the gB promoter-leader sequence was ligated into the *Bst*EII site of pRB4342, and the *Sal*I-*Eco*RI fragment was cloned into the *Sal*I site of pFR400 (35) to generate pRB4354. The resulting hybrid gene has a gB promoter-leader sequence, the $U_S11$ open reading frame, and the α47-$U_S11$-$U_S10$ polyadenylation signal. This plasmid also encodes a truncated $U_S10$ open reading frame which may be transcribed from its own promoter. (F) Photographic image of a nitrocellulose blot of electrophoretically separated proteins from methotrexate-resistant clones probed with anti-$U_S11$ monoclonal antibody.

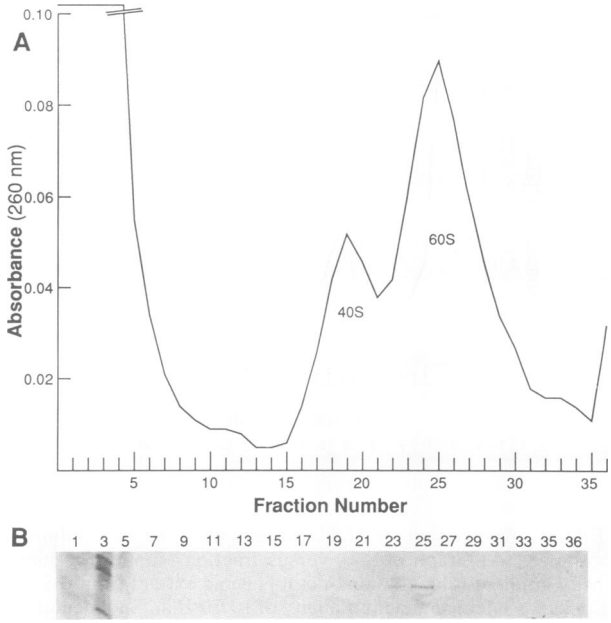


FIG. 8. Association of $U_S11$ protein in $U_S11$cl19 cells with the 60S subunit of ribosomes. (A) Graph of $A_{260}$ versus fraction number for sucrose density gradient fractionation of cytoplasmic extract from ~$10^8$ $U_S11$cl19 cells treated with 250 μM puromycin for 30 min before harvesting. Sedimentation is from left to right. One-fifteenth of each fraction was used for determination of absorbance. (B) Photographic image of a blot of electrophoretically separated polypeptides from odd-numbered fractions (and including the pellet fraction, 36) of the gradient depicted in panel A, probed with anti-$U_S11$ monoclonal antibody. Only the portion of the blot around the $M_r$ of $U_S11$ protein is shown.

latter contention, however, requires purification of the fusion protein to homogeneity.

(ii) We have previously shown that small additions to the carboxyl terminus of $U_S11$ do not impair its RNA binding activity (31). We showed here that the RNA binding activity can also tolerate an enormous addition to the amino terminus of $U_S11$ protein.

**$U_S11$ protein as a virion component.** The conclusion that $U_S11$ protein is a component of the HSV-1 virion rests on three lines of evidence: (i) $U_S11$ protein was detected among electrophoretically separated polypeptides from purified HSV-1(F) virions by immunoblotting; (ii) a protein with an $M_r$ characteristic of $U_S11$ protein was readily detected by staining of total HSV-1(F) virion proteins separated in denaturing gels but was undetectable in protein from purified R3631 ($U_S11^-$) virions; (iii) $U_S11$ was readily detected by immunoblotting in cytoplasmic extracts of cells infected for 2 h in the presence of phosphonoacetic acid. The presence of significant amounts of $U_S11$ protein in the virion suggests that it has a regulatory role in the early phases of infection in a manner similar to that of the virion host shutoff factor (*vhs*), which causes degradation of RNA (7, 8, 14, 24) and of the α *trans*-inducing factor which induces the expression of α genes (2, 3, 16, 17, 25, 27). Experiments are under way to determine whether $U_S11$ protein binds to or alters the function of cellular or viral RNAs during the early phases of infection.

In addition to being a passenger, the $U_S11$ protein may serve some function in the assembly or maturation of the virus particle. Inasmuch as it is dispensable for replication in

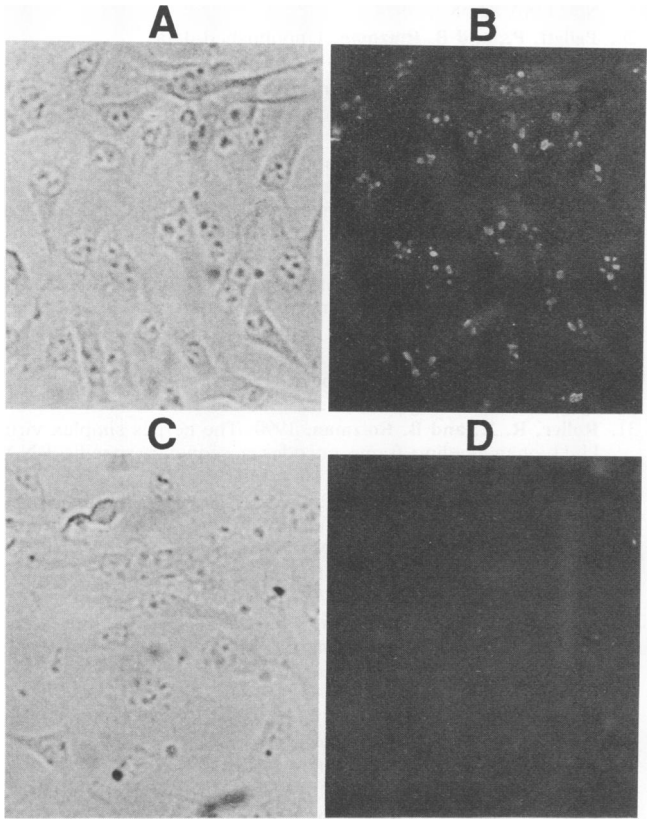


FIG. 9. Localization of $U_S11$ protein to the nucleoli of $U_S11cl19.1$ cells. (A) Photographic image of $U_S11cl19.1$ cells reacted with anti-$U_S11$ monoclonal antibody and fluorescein isothiocyanate-conjugated secondary antibody viewed with phase-contrast optics. (B) Photographic image of cells shown in panel A, viewed with UV illumination. (C) As in panel A, but α4cl13 cells are shown. (D) Photographic image of cells shown in panel C, viewed with UV illumination.

cell culture, it is unlikely that $U_S11$ encodes an essential structural protein. We have not determined the virion compartment in which $U_S11$ protein is contained. We believe it likely, however, that it is in the tegument, since $U_S11$ is not a membrane-associated protein, and since it seems unlikely that the highly ordered capsid contains any protein dispensable for viral growth in cell culture. Protein with an $M_r$ similar to $U_S11$ has not been reported in capsid preparations (9, 10).

**Association of $U_S11$ protein with ribosomes.** We showed that $U_S11$ protein associates specifically with ribosomal 60S subunits in cytoplasmic extracts of infected cells. Because free $U_S11$ is present in the cytoplasm (Fig. 5B), and because $U_S11$ protein does not bind the 40S component, we conclude that the association with 60S subunits and intact ribosomes is specific. The association of $U_S11$ protein with nucleoli in infected cells suggests strongly that $U_S11$ association with ribosomal components also occurs in the intact infected cell and is not an artifact of the extraction and centrifugation procedure. The most economical hypothesis is that the localization of $U_S11$ to nucleoli and the cosedimentation of $U_S11$ protein with 60S subunits and intact ribosomes and polysomes are all due to a specific affinity of $U_S11$ for some component of the 60S ribosomal subunit. The RNA binding activity of $U_S11$ protein is suggestive of an interaction with 28S rRNA, and experiments are in progress to test for specific binding.

To our knowledge, $U_S11$ protein is the first HSV-encoded protein shown to associate with ribosomal components in the infected cell. Masse et al. (20) detected three novel phosphoproteins which associated with ribosomal subunits and monosomes in extracts of infected cells. Their viral origin was not documented, however, and none of these matches the $U_S11$ protein with respect to size, kinetics of appearance, or distribution among ribosomal subunits.

Two issues are relevant to the association of $U_S11$ protein with the 60S ribosomal subunit, ribosomes, and nucleoli. First, one of the features of HSV-1 infection of permissive cells is the disaggregation of nucleoli (34) and polysomes (37, 38) during infection and cessation of rRNA synthesis (39). Work in progress suggests that at least the former two processes are not dependent on $U_S11$ protein since they occur also in cells infected with $U_S11^-$ virus.

We have previously shown that $U_S11$ protein regulates the steady-state level of a viral RNA late in infection (32). Perhaps the most intriguing question raised by these studies is whether that event is related to the associations of $U_S11$ protein with virions and ribosomal subunits documented here.

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## REFERENCES

1. **Arsenakis, M., J. Hubenthal-Voss, G. Campadelli-Fiume, L. Pereira, and B. Roizman.** 1986. Construction and properties of a cell line constitutively expressing the herpes simplex virus glycoprotein B dependent on functional α4 protein synthesis. J. Virol. **60**:674–682.
2. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. J. Virol. **46**:371–377.
3. **Campbell, M. E. M., J. W. Palfreyman, and C. M. Preston.** 1984. Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. J. Mol. Biol. **180**:1–19.
4. **Challberg, M. D.** 1986. A method for identifying the viral genes required for herpesvirus DNA replication. Proc. Natl. Acad. Sci. USA **83**:9094–9098.
5. **Cullen, B. R., and W. C. Greene.** 1990. Functions of the auxiliary gene products of the human immunodeficiency virus type 1. Virology **178**:1–5.
6. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. J. Gen. Virol. **2**:357–364.
7. **Fenwick, M. L.** 1984. The effects of herpesviruses on cellular macromolecular synthesis. Compr. Virol. **19**:359–390.
8. **Fenwick, M. L., and M. J. Walker.** 1978. Suppression of the synthesis of cellular macromolecules by herpes simplex virus. J. Gen. Virol. **41**:37–51.
9. **Gibson, W., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. **10**:1044–1052.
10. **Gibson, W., and B. Roizman.** 1974. Proteins specified by herpes simplex virus. X. Staining and radiolabeling properties of B capsid and virion proteins in polyacrylamide gels. J. Virol. **13**:155–165.
11. **Heine, J. W., R. W. Honess, E. N. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J. Virol. **14**:640–651.

12. **Johnson, P. A., C. MacLean, H. S. Marsden, R. G. Dalziel, and R. D. Everett.** 1986. The product of gene $U_S11$ of herpes simplex virus type 1 is expressed as a true late gene. J. Gen. Virol. **67:**871–883.
13. **Köhler, G., and C. Milstein.** 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur. J. Immunol. **6:**511–519.
14. **Kwong, A. D., J. A. Kruper, and N. Frenkel.** 1988. Herpes simplex virus virion host shutoff function. J. Virol. **62:**912–921.
15. **Lemke, H., G. J. Hämmerling, C. Höhmann, and K. Rajewski.** 1978. Hybrid cell lines secreting monoclonal antibody specific for major histocompatibility antigens of the mouse. Nature (London) **271:**249–251.
16. **Mackem, S., and B. Roizman.** 1982. Regulation of α genes of herpes simplex virus: the α27 gene promoter-thymidine kinase chimera is positively regulated in converted L cells. J. Virol. **43:**1015–1023.
17. **Mackem, S., and B. Roizman.** 1982. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. **44:**939–949.
18. **MacLean, C. A., F. J. Rixon, and H. S. Marsden.** 1987. The products of gene $U_S11$ of herpes simplex virus type 1 are DNA-binding and localize to the nucleoli of infected cells. J. Gen. Virol. **68:**1921–1937.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Masse, T., D. Garcin, B. Jaquemont, and J.-J. Madjar.** 1990. Ribosome and protein synthesis modifications after infection of human epidermoid carcinoma cells with herpes simplex virus type 1. Mol. Gen. Genet. **220:**377–388.
21. **Mavromara-Nazos, P., M. Ackermann, and B. Roizman.** 1986. Construction and properties of a viable herpes simplex virus 1 recombinant lacking the coding sequences of the α47 gene. J. Virol. **60:**807–812.
22. **McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon.** 1986. Sequence determination and genetic content of the short unique region of the genome of herpes simplex virus type 1. J. Mol. Biol. **181:**1–13.
23. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Nishioka, Y., and S. Silverstein.** 1977. Degradation of cellular mRNA during infection by herpes simplex virus. Proc. Natl. Acad. Sci. USA **74:**2370–2374.
25. **Pellett, P., J. L. C. McKnight, F. J. Jenkins, and B. Roizman.** 1985. Nucleotide sequence and predicted amino acid sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of trans-inducing α genes. Proc. Natl. Acad. Sci. USA **82:**5870–5874.
26. **Pellett, P., and B. Roizman.** Unpublished data.
27. **Post, L. E., S. Mackem, and B. Roizman.** 1981. Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. Cell **24:**555–565.
28. **Purves, F. C., D. Spector, and B. Roizman.** 1991. The herpes simplex virus 1 protein kinase encoded by the $U_S3$ gene mediates posttranslational modification of the phosphoprotein encoded by the $U_L34$ gene. J. Virol. **65:**5757–5764.
29. **Rixon, F. J., and D. J. McGeoch.** 1984. A 3′ co-terminal family of mRNAs from the herpes simplex virus type 1 short region: two overlapping reading frames encode unrelated polypeptides one of which has a highly reiterated amino acid sequence. Nucleic Acids Res. **12:**2473–2587.
30. **Roizman, B., and P. G. Spear.** 1968. Preparation of herpes simplex virus of high titer. J. Virol. **2:**83–84.
31. **Roller, R. J., and B. Roizman.** 1990. The herpes simplex virus $U_S11$ open reading frame encodes a sequence-specific RNA-binding protein. J. Virol. **64:**3463–3470.
32. **Roller, R. J., and B. Roizman.** 1991. Herpes simplex virus 1 RNA binding protein $U_S11$ negatively regulates the accumulation of a truncated viral mRNA. J. Virol. **65:**5873–5879.
33. **Rüther, U., and B. Müller-Hill.** 1983. Easy identification of cDNA clones. EMBO J. **2:**1791–1794.
34. **Schwartz, J., and B. Roizman.** 1969. Similarities and differences in the development of laboratory strains and freshly isolated strains of herpes simplex virus in HEp-2 cells: electron microscopy. J. Virol. **4:**879–889.
35. **Simonsen, C. C., and A. D. Levinson.** 1983. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. USA **80:**2495–2499.
36. **Spear, P., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. **9:**143–159.
37. **Sydiskis, R. J., and B. Roizman.** 1966. Polysomes and protein synthesis in cells infected with a DNA virus. Science **153:**76–78.
38. **Sydiskis, R. J., and B. Roizman.** 1968. The sedimentation profiles of cytoplasmic polyribosomes in mammalian cells productively and abortively infected with herpes simplex virus. Virology **34:**562–565.
39. **Wagner, E. K., and B. Roizman.** 1969. Ribonucleic acid synthesis in cells infected with herpes simplex virus. I. Patterns of ribonucleic acid synthesis in productively infected cells. J. Virol. **4:**36–46.
40. **Watson, R. J., and G. F. Vande Woude.** 1982. DNA sequence of an immediate early gene (IE mRNA-5) of herpes simplex virus type 1. Nucleic Acids Res. **10:**979–991.