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Identification of Saint Louis Encephalitis Virus mRNA

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Saint Louis encephalitis (SLE) virus-specific RNA was recovered from infected HeLa cells by sodium dodecyl sulfate (SDS)-phenol-chloroform extraction, and the molecular species were resolved by SDS-sucrose gradient centrifugation and agarose gel electrophoresis. Sucrose gradient centrifugation revealed the presence of a 45S species, minor 20 to 30S heterogeneous species, and an 8 to 10S RNA species in the cytoplasmic extract. Analysis of the same samples by electrophoresis on agarose gels, under both nondenaturing and denaturing conditions, revealed only two virus-specific RNA molecules, the 45S genome-sized RNA and an 8 to 10S species. Varying the gel concentration to facilitate analysis of nucleic acids with molecular weights ranging from 25,000 to 25×10^6 failed to reveal additional RNA species, although low levels of a putative double-stranded replicative form could conceivably have escaped detection. From our observations it appears that the heterogeneous RNA species and presumably the 20S RNase-resistant species reported in other investigations of flavivirus RNA are degradation products or conformers of the 45S molecule. Polysomes from SLE virus-infected cells were prepared and separated from contaminating nucleocapsid by centrifugation on discontinuous sucrose gradients. RNA extracted from these polysome preparations was analyzed by sucrose gradient centrifugation and agarose gel electrophoresis. The 45S SLE virus genome-size molecule was found to be the only RNA species associated with the polysomes. This molecule was sensitive to RNase digestion and was released from polysomes by EDTA and puromycin treatment. These findings provide direct evidence that the 45S SLE virus RNA serves as the messenger during virus replication, in contrast to the 26S RNA species which functions as the predominant messenger during alphavirus replication.

Saint Louis encephalitis (SLE) virus is a flavivirus that is thought to mature in association with internal membranes of the rough endoplasmic reticulum (5, 11). The virion is composed of three structural proteins designated p-7(M), p-14(N), and gp-53(E) (25). Proteins p-7 and gp-53 are found in the virion envelope, and p-14 is associated with the nucleocapsid (28). Viral structural protein gp-53 is glycosylated (19), stimulates the formation of antibodies (15), and functions as the viral hemagglutinin (12). Westaway (29) has identified seven nonstructural polypeptides in SLE virus-infected cells, comprising a total of 370,000 daltons of virus-specified protein. In a study with a related flavivirus, Kunjin, it was suggested that neither the structural nor the nonstructural proteins are processed by post-translational cleavage (30). When infected cultures were treated with protease inhibitors tolysulfonyl phenylalanyl chloromethyl ketone and *N*-tosyl-L-lysyl chloromethane, high and low temperatures of incubation, and amino acid analogs, no precursor polyprotein could be demonstrated. The use of short pulse-chase conditions did reveal what appeared to be minor processing of the smaller flavivirus-specific proteins NV-2, NV-2½, and NV-1. Short pulse-chase experiments with labeled amino acids under conditions of synchronized initiation revealed that the synthesis of all virus-specific proteins was completed within 9 min of initiation. This period of time is much too brief for synthesis of a polyprotein with post-translational cleavage such as that found in alphavirus or picornavirus infection (6, 18). These results have been confirmed in our laboratory with SLE virus (D. W. Trent and C. W. Naeve, unpublished data). It thus appears that SLE virus and other flaviviruses have a unique method of gene expression. Either several monocistronic viral mRNA's function to code for individual proteins, or a single polycistronic mRNA with multiple internal initiation and termination sites codes for all of the virus proteins.

The SLE virus single-stranded genome RNA

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(43S, 3.3 × 10^6 daltons) is infectious and therefore presumably of the same polarity as mRNA (27). It is of sufficient length to code for 370,000 daltons of protein. In addition to the 43S genome-sized RNA, three other species of RNA (26S, 20S, and 8 to 10S) have been found in flavivirus-infected cells by sucrose gradient analysis (9, 22, 24, 27, 32). The 20S species is RNase resistant and presumably double stranded. By agarose gel electrophoresis we established that only 45S and 8 to 10S viral RNAs are present in infected HeLa cells and that the 45S viral RNA is the only species associated with polysomes and presumably functions as the viral mRNA. These observations support Westaway's hypothesis of multiple internal initiation sites and demonstrate that flavivirus gene expression is unlike that of other plus-stranded RNA viruses.

(This report is from a thesis submitted to the Graduate Faculty of Colorado State University in partial fulfillment of the Ph.D. degree requirements.)

MATERIALS AND METHODS

Virus production and cell culture. Stock suspensions of TBH-28 SLE virus were prepared in 3-day-old suckling mice. Three days postinfection the mice became paralyzed and were sacrificed, and their brains were harvested with an 18-gauge needle and syringe and homogenized in a Bellco glass homogenizer. A 10% (wt/vol) suspension of infected mouse brain was prepared in Dulbecco modified Eagle medium (DME) supplemented with 20% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 mg/ml), and Fungizone (0.25 mg/ml). This suspension was centrifuged at 10,000 × *g* for 30 min to remove cell debris, and the supernatant was dispensed in 1-ml portions and stored at −70°C. The titer of this suspension was 8 × 10^7 PFU/ml.

Plaque assays of viral infectivity were performed in BHK-21/15 cells (8). Virus was allowed to adsorb for 1 h at 37°C, and an overlay was made with 3 ml of medium composed of medium 199, 2.5% FCS, 0.5% agarose, and the antibiotics penicillin (100 U/ml), streptomycin (100 U/ml), and mycostatin (0.25 mg/ml). The plates were incubated at 37°C under 5% CO_2 and read after 3 to 4 days by staining with neutral red (0.04 g/liter) incorporated into a second overlay.

All experimental work was carried out with the Ohio strain of HeLa cells (Flow Laboratories). These cells were grown in DME medium supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Cells were grown to confluency in 3 days in 150-cm^2 plastic tissue culture flasks (Corning) and contained approximately 4 × 10^7 cells as determined by hemocytometer count.

SLE virus produced in HeLa cell cultures was purified by polyethylene glycol precipitation followed by velocity sedimentation in sucrose and isopycnic centrifugation in potassium tartrate as previously described (25).

Polysome preparation. Cells were infected with SLE virus at a multiplicity of infection (MOI) of 2.0, and the virus was allowed to adsorb at 37°C for 60 min before fresh DME containing 10% FCS and antibiotics was added. At 12 h postinfection, medium containing actinomycin D (ACD, 4 μg/ml) was added to inhibit host cell DNA-dependent RNA synthesis, and 1 h later [5-^{3}H]uridine (New England Nuclear, 100 μCi/ml) was added to the culture medium. At 17 h and 20 min postinfection, cycloheximide (CH) was added to a concentration of 4 μg/ml to prevent ribosome translocation. At 18 h postinfection, cells were harvested and a cytoplasmic extract was prepared as described by Penman et al. (12). Approximately 4 × 10^7 cells were rinsed with cold phosphate-buffered saline, and the cells were scraped from the flask surface with a rubber policeman. The cells were washed twice by centrifuging them at 1,800 rpm at 4°C for 3 min in conical tubes in an IEC-R2 centrifuge. The cell pellet was suspended in 2 ml of reticulocyte standard buffer (RSB; 10 mM Tris-hydrochloride [pH 7.4]–10 mM NaCl–3 mM $MgCl_2$) and allowed to swell for 10 min. The cells were lysed by adding 0.2 ml of a 1:1 mixture of 10% deoxycholate and 10% Nonidet P-40, and the mixture was centrifuged for 2 min at 2,000 rpm at 4°C in the IEC-R2 centrifuge. Nuclei were quantitatively pelleted by this procedure as revealed by trypan blue exclusion or observation with phase-contrast optics.

The supernatant was placed on a 10 to 40% sucrose-RSB rate zonal gradient and centrifuged at 22,500 rpm for 130 min at 4°C in the SW-27 rotor (Beckman Instruments). Fractions were collected from the bottom of the gradient, and the absorbance at 260 nm (A_{260}) was measured in the flow cell of a Beckman 25 recording spectrophotometer. Portions of each fraction were assayed for trichloroacetic acid-precipitable radioactivity by filtration through 0.45-μm membranes (Millipore HA). The filters were washed twice with 7% trichloroacetic acid and once with 95% ethanol, dried, and counted in 10 ml of Econofluor scintillation fluid (New England Nuclear) in a Beckman LS-250 liquid scintillation spectrophotometer.

Isopycnic centrifugation of polysomes. Isopycnic centrifugation of glutaraldehyde-fixed polysomes was carried out in CsCl gradients as described by Baltimore and Huang. (2). Polysomes prepared by rate zonal centrifugation on sucrose gradients were pooled, diluted with RSB, and pelleted by centrifugation at 50,000 rpm for 3 h at 4°C in a Beckman 42.1 fixed-angle rotor. The polysome pellet was suspended in 2 ml of RSB and was either treated with EDTA (10 mM) or treated with RSB. To this mixture 0.525 ml of 40% (wt/vol) neutralized glutaraldehyde was added, and the suspension was applied to a 10-ml CsCl gradient prepared in RSB (1.38 to 1.56 g/cm^3). The gradient was centrifuged at 37,500 rpm for 7.5 h at 4°C, and fractions were collected from the bottom of the gradient and assayed for trichloroacetic acid-precipitable radioactivity as described above.

Discontinuous centrifugation of polysomes. The isolation of SLE virus-infected HeLa cell polysomes free of contaminating nucleocapsid and nonpolysomal-associated viral RNA was carried out by centrifuging cytoplasmic extracts on a discontinuous

sucrose gradient in a modification of the method described by Blobel (3). Cytoplasmic extract was applied to a discontinuous gradient composed of 4 ml of 0.5 M sucrose in TKM buffer (0.05 M Tris-hydrochloride [pH 7.4]–0.15 M KCl–0.005 $MgCl_2$) over 3 ml of 2.0 M sucrose in TKM buffer. The gradient was centrifuged at 24,500 rpm for 2.5 h at 4°C in the SW-41 rotor, and the pelleted ribosomes were stored frozen at −20°C.

Puromycin and EDTA release of polysome-associated RNA. A procedure described by Blobel and Sabatini (4) in which the aminoacyl-tRNA analog puromycin effects dissociation of polysomes into subunits was used to release mRNA. Infected-cell polysomes were suspended in distilled water to 195 A_{260} units/ml. To 0.2 ml of this suspension, 0.25 ml of compensating buffer (1 M KCl–0.1 M Tris-hydrochloride [pH 7.5]–0.01 M $MgCl_2$) and 0.05 ml of 0.01 M puromycin at pH 7.0 was added. This mixture was incubated at 0°C for 15 min and then at 37°C for 10 min. The sample was applied to a 5 to 20% (wt/vol) sucrose rate zonal gradient prepared in TKMP buffer (500 mM KCl–50 mM Tris-hydrochloride [pH 7.5] -and 5 mM $MgCl_2$). The gradients were centrifuged at 39,000 rpm for 110 min at 20°C in the SW-41 rotor, and fractions were collected from the bottom of the gradient and monitored for A_{260}. Samples were assayed for trichloroacetic acid-precipitable radioactivity.

Release of polysome-associated RNA was also effected by EDTA dissociation of purified ribosomes. The cytoplasmic extract to be analyzed was made 10 mM with EDTA and layered on a 10 to 40% (wt/vol) sucrose gradient prepared in TKMP buffer. Gradients were centrifuged at 22,500 rpm for 130 min at 4°C in the SW-27 rotor. Fractions were collected from the bottom of the gradient and monitored for A_{260} and for trichloroacetic acid-precipitable radioactivity.

Isolation and analysis of viral RNA. RNA was extracted from purified virus, cytoplasmic extract, or whole cells as follows. The material to be extracted was suspended in RSB containing 1% sodium dodecyl sulfate (SDS) and incubated at 37°C for 10 min. An equal volume of phenol extraction mixture (475 ml of water-saturated phenol, 70 ml of *m*-cresol, 0.6 g of 8-hydroxyquinoline, and 200 ml of chloroform) at room temperature was added, the solution was shaken vigorously for 1 to 2 min, and the aqueous and phenol phases were resolved by centrifugation at 14,000 rpm in a Beckman microfuge. The phenol phase was removed, and the interface and aqueous phases were re-extracted until little or no visible interface remained. The aqueous phases were made 0.2 M with LiCl, and the RNA was precipitated with 2 volumes of 95% ethanol at −20°C. The precipitate was recovered by centrifugation at 14,000 rpm for 2 min in a Beckman microfuge. The precipitate was suspended in the appropriate buffer for further analysis.

RNA concentrations were determined by monitoring A_{260}, by using the concentration mode of the Beckman model 25 spectrophotometer, or by the original colorimetric procedure (20).

RNase sensitivity of viral RNA from virions or polysome samples was accomplished by suspending the RNA in RSB, adding pancreatic RNase (Calbiochem) to 0.5 μg/ml, and allowing incubation at 22°C for 30 min. The RNase was removed by extraction with phenol as described above.

Rate zonal centrifugation analysis of RNA samples was carried out on preformed linear sucrose gradients (10 to 30%, wt/vol) in TNE buffer (0.01 M Tris-hydrochloride–0.1 M NaCl–0.001 M EDTA, pH 7.4) containing 0.5% SDS. The sample, resuspended in TNE with 0.5% SDS, was applied to a 13.8-ml gradient and centrifuged at 18,000 rpm for 17 h at 22°C in the SW-41 rotor. Fractions were collected from the bottom of the gradient and assayed for A_{260} and for radioactivity.

Viral RNA was also analyzed by agarose gel electrophoresis as described by Weil (28) and Rosen et al. (17). RNA samples to be analyzed under nondenaturing conditions were resuspended in TEB buffer, pH 8.3 (90 mM Tris–2.5 mM EDTA–90 mM boric acid), containing 20% RNase-free sucrose and 0.005% bromophenol blue (BPB). RNA was electrophoresed on 23-cm gel tubes containing 1.5% (wt/vol) agarose in TEB. The gels were prerun at 1 mA/gel for 10 min, and the sample was applied and allowed to electrophorese at 1 mA/gel for 6 h in TEB buffer at 4°C.

RNA to be analyzed under denaturing conditions was suspended in CA buffer (0.025 M citric acid, adjusted to pH 3.5 with saturated NaOH) containing 20% (wt/vol) sucrose and 0.005% BPB. Twenty-three-centimeter gels of 1.5% (wt/vol) agarose in CA buffer containing 6 M urea were prerun at 2 mA/gel for 10 min. Samples were electrophoresed at 2 mA/gel until the BPB dye marker was 4 cm from the bottom. The gels were removed, fixed in 7% trichloroacetic acid for 30 min at 4°C, manually sliced into 2-mm sections, dissolved in Econofluor containing 4% TS-1 solubilizer (Research Products International), and counted in the Beckman LS-250 liquid scintillation spectrometer.

RESULTS

Growth and RNA synthesis of SLE virus. The growth curve of SLE virus in HeLa-O cells infected at an MOI of 2.0 is presented in Fig. 1. The virus replicated in these cells to titers comparable with those observed in other more commonly used cell lines. Maximum titers of infectious virus (2×10^7 PFU/ml) were reached 17 h after infection and continued at this level for an additional 18 h. A latent phase requiring 9 h is characteristic of SLE virus replication in these cells. Viral RNA synthesis, as measured by the incorporation of [^{3}H]uridine into trichloroacetic acid-precipitable material, proceeds in a biphasic manner. The first burst of viral RNA synthesis occured at 9 h postinfection and was followed by a second surge of synthesis beginning at 16 h postinfection, increasing to reach maximum levels at 24 h postinfection before declining with the onset of cytopathic effects. The replication of SLE virus in HeLa-O cells under these conditions of infection after ACD treatment (4 μg/ml) at 12 h postinfection was similar.

Intracellular SLE virus-specific RNA. To determine the species of viral RNA present in

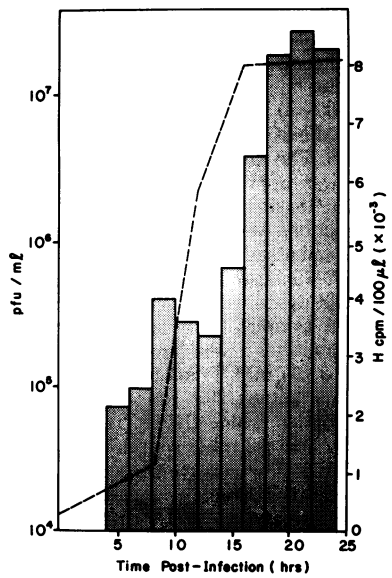

FIG. 1. *Growth of SLE virus in HeLa-O cells. Replicate cultures of cells were treated with ACD (1 µg/ml) for 1 h before infection, and at 2-h intervals after infection cultures were pulse-labeled with [³H]uridine (10 µCi/ml) for 30 min. The cells were washed with saline and solubilized in 1% (wt/vol) SDS, a 100-µl portion of the solubilized material was trichloroacetic acid precipitated, the precipitate was collected on filters, and radioactivity was counted as described in Materials and Methods. (- - -) PFU/ml; (——) ³H counts per minute per 100 µl.*

SLE virus-infected HeLa cells, the infected cells were solubilized with SDS at 18 h postinfection, and the RNA was extracted. The RNA was analyzed by both agarose gel electrophoresis and sucrose gradient centrifugation. Viral RNA prepared in this fashion exhibits a heterogeneous profile when displayed on sucrose gradients (Fig. 2). The largest molecule, identical to virion RNA, migrated at 45S, with a smaller species sedimenting at 8 to 10S. Two peaks containing a relatively small amount of radioactivity migrated in the 20 to 30S range. Analysis of intracellular viral RNA in these same samples on 1.5% agarose gels under denaturing conditions revealed markedly different results (Fig. 3A). In this profile only two virus-specific molecules are observed: a 45S peak corresponding in size to genome RNA and an 8 to 10S peak migrating slightly behind the BPB marker dye. The apparent molecular weights of these viral RNAs were determined by coelectrophoresis with cellular 28S, 18S, and 4S RNA species and were found to be 3.98×10^6 and 80×10^3, respectively. When the RNA was extracted from cytoplasmic extracts from which the nuclei had been removed, only the 45S molecule was observed (Fig. 3B).

Although the 8 to 10S species comprises the majority of the RNA observed in infected cells under these conditions and is apparently associated with nuclei, its role in SLE virus replication is unknown. Both the 45S and 8 to 10S RNA species present in whole-cell extracts are RNase sensitive (Fig. 3C). Since the 26S and 20S species seen in previous studies (9, 11, 24, 27, 31) were not observed when analyzed under denaturing conditions, these samples were electrophoresed in agarose gels in TEB buffer without urea. The results obtained by electrophoresis under nondenaturing conditions were virtually identical to those presented in Fig. 3 (data not shown). Since it has been suggested that the 20S molecule is a double-stranded 45S species (22), repeated attempts were made to resolve such a molecule ($\sim 8 \times 10^6$ daltons) on 1.0 and 0.5% agarose gels.

Electrophoresis of the intracellular SLE RNA failed to reveal a peak of radioactivity corresponding to the position in the gels at which molecules of approximately 7.2×10^6 daltons would migrate. The presence of a few copies of molecules of this size could not be excluded (data not shown).

Infected-cell polysome preparation. Polysomes from SLE virus-infected and uninfected HeLa cells were prepared and analyzed by sedimentation on 10 to 40% sucrose gradients (Fig. 4A through D). Preparation of cytoplasmic extracts without prior CH treatment resulted in a relatively poor yield of polysomes, presumably

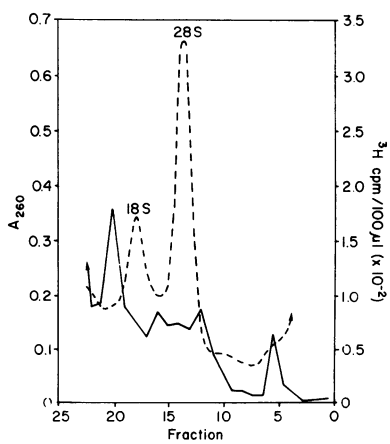

FIG. 2. *Rate zonal sucrose centrifugation of intracellular, SLE virus-specific, trichloroacetic acid-precipitable RNA. Phenol-SDS-extracted RNA was suspended in TNE with 0.5% SDS, layered over a 10 to 40% sucrose-TNE gradient with 0.5% SDS, and centrifuged for 17 h at 18,000 rpm (22°C) in an SW-41 rotor. (- - -) A_{260} of 28S and 18S ribosome markers; (——) ³H counts per minute per 100 µl of SLE virus RNA. Sedimentation is from left to right.*

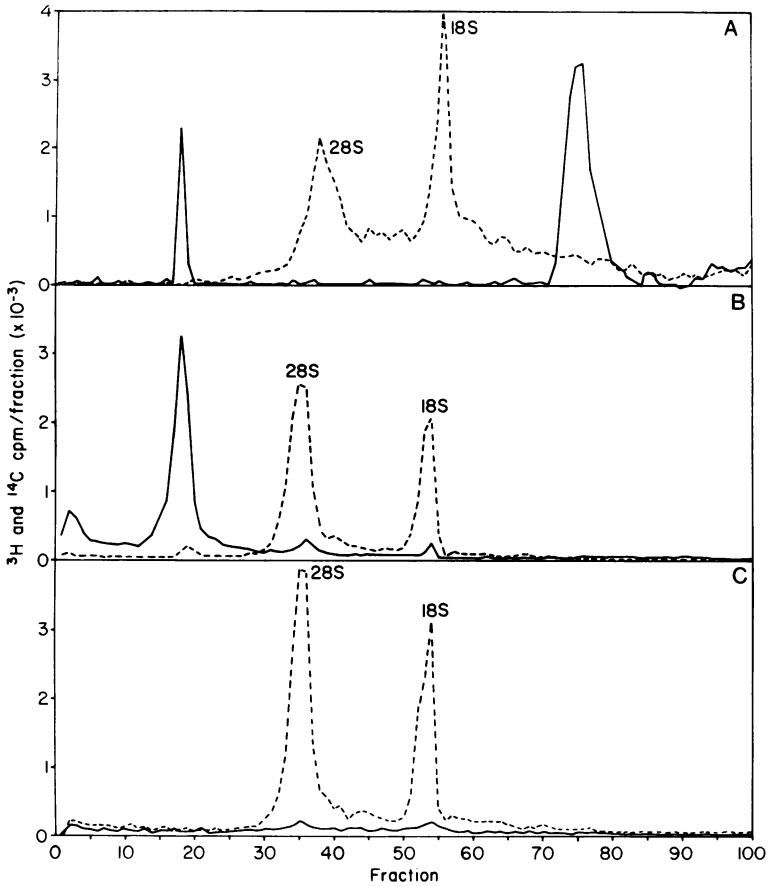

FIG. 3. *Coelectrophoresis of intracellular [^{3}H]uridine-labeled SLE virus RNA and [^{14}C]uridine-labeled 28S and 18S ribosome markers. Electrophoresis in 1.5% agarose gels was performed under denaturing conditions as described in Materials and Methods. (A) SLE virus intracellular RNA extracted from whole cells. (B) SLE virus intracellular RNA extracted from cytoplasmic extracts. (C) RNase treatment of SLE virus RNA from whole cells. (- - -) ^{14}C counts per minute per fraction; (——) ^{3}H counts per minute per fraction. Electrophoresis is from right to left in this and all other figures.*

due to ribosomal "runoff" during manipulation of the sample. Treatment of the cell monolayers with CH (4 μg/ml) for varying periods of time prior to harvest inhibited translocation and effectively prevented runoff (Fig. 4A). As the time of CH exposure increased to 40 min the yield of polysomes also increased. Treatment for longer periods of time did not substantially increase the polysome yield, indicating that treatment with 4 μg of CH per ml for 40 min saturated the mRNA with ribosomes. This level of CH exposure resulted in a noticeable shift in sedimentation rate of polysomes toward the bottom of the gradient.

To demonstrate that the [^{3}H]uridine-labeled material sedimenting in the polysomal region of the gradient was ribosome associated, the sensitivity of the material to EDTA and RNase was determined. The divalent ion-chelating agent EDTA is routinely used to dissociate polysomes (1). As seen in Fig. 4B, the polysomes in SLE virus-infected-cell cytoplasmic extracts were dissociated by both EDTA treatment and RNase digestion.

The effect of ACD treatment on the polysomal profile of SLE virus-infected HeLa cells was determined (Fig. 4C). Due to the relatively long (24 h) half-life of some HeLa cell mRNA's (14), the level of polysomes determined by optical density is reduced but not completely eliminated by the ACD treatment. The incorporation of [^{3}H]uridine into host cell polysome RNA, however, is negligible.

A comparison of the polysome profiles of uninfected cells and SLE virus-infected cells (Fig. 4D) revealed two points of interest. First, the overall size of polysomes from the HeLa cells is not substantially changed after SLE virus infec-

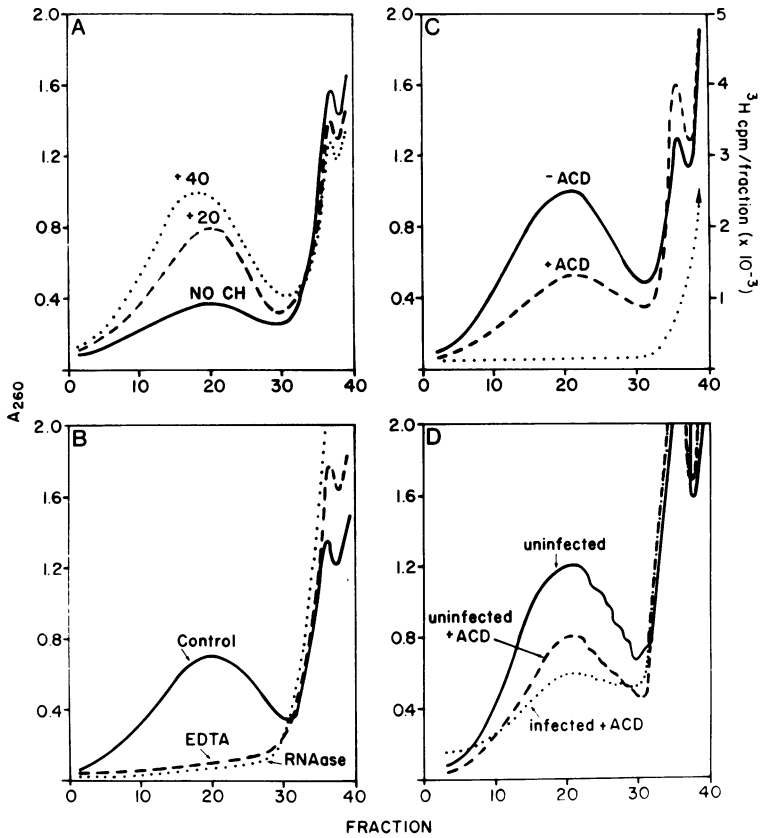

FIG. 4. *Sucrose rate zonal sedimentation of polysomes isolated from HeLa-O cells under various conditions. (A) Effect of treating cells with CH (4 μg/ml) for 20 min (- - -), 40 min (· · ·), or no treatment prior to harvest (——). (B) Effect of EDTA and RNase on infected-cell polysomes: untreated control (——), 10 mM EDTA (- - -), and treatment with 0.5 μg of RNase (· · ·) per ml. (C) Effect of ACD on polysome profile. Optical density profile of polysomes from cells treated with 4 μg of ACD (- - -) per ml, no treatment (——), and [3H]uridine incorporation into cellular polysomes treated with ACD (· · ·). (D) Comparison of sedimentation profiles of polyribosomes from uninfected (——), uninfected ACD-treated (- - -), and SLE virus-infected ACD-treated cells (· · ·). Sedimentation is from right to left in this and all other polysome profile patterns.*

tion. This is in contrast to results seen in poliovirus-infected cells (23) and probably reflects the fact that flaviviruses do not inhibit host cell protein synthesis (27). Second, there was a marked increase in A_{260} at the heavy end of the gradient and a n increase in material sedimenting at approximately 140S, indicating that after SLE virus infection there was an apparent broadening of the polysome profile.

Elimination of contaminating nucleocapsid. To characterize the A_{260}-absorbing material sedimenting in the 140S to 160S portion of the gradient, cultures were infected with virus, treated with ACD, and labeled for 5 h with [3H]uridine before cytoplasmic extracts were prepared. Analysis of the virus-specific material sedimenting in rate zonal sucrose gradients is presented in Fig. 5A. A pronounced peak of [3H]uridine activity sedimented at approximately 140S. Treatment of the cytoplasmic extract with 10 mM EDTA before it was centrifuged did not alter the material in the 140S peak, indicating that this RNA is not polysome associated. To further analyze cytoplasmic extracts of SLE virus-infected cells, material from the polysomal portion of the gradient was fixed with glutaraldehyde and analyzed on CsCl gradients (2). As seen in Fig. 3B, polysomes sedimented at 1.55 g/cm^3, and the "contaminate" banded at a density of 1.39 g/cm^3. Treatment of the cytoplasmic extract with EDTA before it was fixed and centrifuged resulted in a 50% reduction in the level of polysomal material sedimenting at 1.55 g/cm^3 and the appearance of released RNA, which sedimented at a density of 1.42 to 1.45 g/ml. Under these conditions the

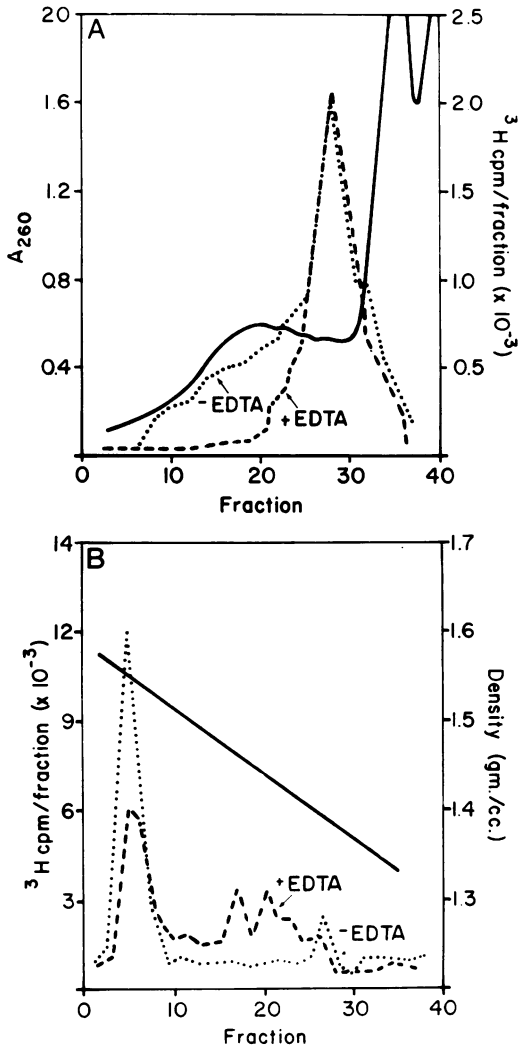

FIG. 5. *Velocity and equilibrium centrifugation of [^{3}H]uridine-labeled SLE virus intracellular RNA in sucrose and CsCl gradients, respectively. (A) Distribution of SLE virus-specific intracellular RNA in polysome gradients. (——) A_{260}; (· · ·) ^{3}H counts per minute without EDTA treatment; (- - -) ^{3}H counts per minute with EDTA treatment. (B) Isopycnic (CsCl) centrifugation of glutaraldehyde-fixed infected-cell polysomes with (- - -) and without (· · ·) EDTA treatment. Gradient density is indicated by the solid line.*

material sedimenting at 1.39 was not affected. The density of the [^{3}H]uridine-labeled material that was released from the viral polysomes by EDTA treatment suggested that the released mRNA is associated with protein. The nature of this mRNA-associated protein is not known.

From these experiments it was clear that to identify polysome-associated SLE virus RNA, it would be necessary to eliminate the "contaminate" that has the same size and density as the SLE virus nucleocapsid (26). Isopycnic centrifugation on CsCl gradients effectively resolved the polysomes and nucleocapsid but required glutaraldehyde fixation to prevent ribosome dissociation. Recovering intact RNA from glutaraldehyde-fixed polysomes is difficult (2). Therefore, several other media were investigated as alternates to CsCl in an attempt to separate the contaminate from the ribosomes by isopycnic centrifugation. Metrizamide, Hypaque, Ficol, and sodium diatrizoate were investigated as possible media for isopycnic centrifugation. All of these materials dissociated unfixed polysomes and therefore could not be used. It was subsequently found that the discontinuous sucrose gradient described by Blobel (3) could be used to facilitate separation of the nucleocapsid from polysomal RNA. As seen in Fig. 6A, when the 208S SLE virion was centrifuged through the discontinuous gradient it was retained at the 2.0 M sucrose interface. Non-polysome-associated virus-specific RNA extracted from infected cells remained in the 0.5 M sucrose layer of the discontinuous gradient (Fig. 6B), and

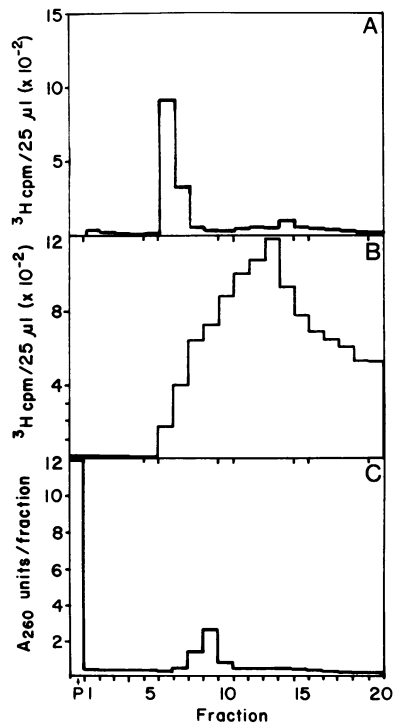

FIG. 6. *Velocity sedimentation of SLE virus, intracellular viral RNA, and polysomes on discontinuous sucrose gradients: SLE virions (A), SLE virus intracellular RNA (B), and HeLa cell polysomes (C).*

polysomes were pelleted during centrifugation (Fig. 6C). Polysomes prepared in this manner were fixed with glutaraldehyde and centrifuged to equilibrium on CsCl gradients described above. The nucleocapsid that sedimented at a density of 1.39 g/cm^3 was absent from the purified polysome preparation (data not shown). This technique clearly separated the 140S nucleocapsid and virus from polysomes and was therefore used in all subsequent experiments.

SLE virus mRNA identification. Infected-cell polysomes were prepared by discontinuous sucrose centrifugation as described, and the virus-specific RNA was analyzed by centrifugation and electrophoresis (Fig. 7). Cosedimentation of [^{3}H]uridine-labeled SLE mRNA and [^{14}C]uridine-labeled uninfected-cell RNA markers revealed that the majority of SLE virus mRNA sediments at 45S. Only a small peak of [^{3}H]-uridine activity was evident at 20S. Electrophoresis of similarly prepared samples on 1.5% agarose gels under nondenaturing conditions revealed that only the 45S molecule was present in polyribosomes from SLE virus-infected cells (Fig. 8).

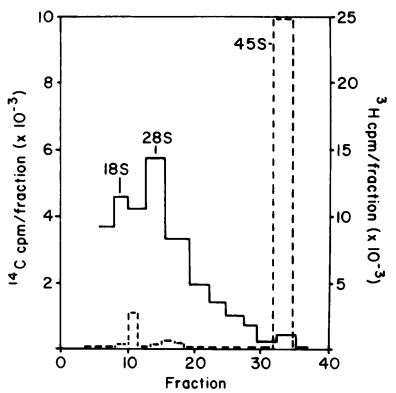

FIG. 7. *Sedimentation of SLE virus polysome-associated RNA in sucrose gradients. RNA was extracted from polysomes prepared as in Fig. 6 and was cosedimented with ribosome 28S and 18S RNA markers (^{14}C).* (——) *^{14}C counts per minute of rRNA;* (- - -) *^{3}H counts per minute of SLE viral RNA.*

Another method was used to confirm the identity of the 45S molecule as mRNA. Puromycin dissociation of ribosomal subunits, as described by Blobel and Sabatini (4), was used to release mRNA from polysome structures (Fig. 9). Centrifugation of polysomes on 5 to 20% sucrose-TKM gradients for 110 min at 39,000 rpm in the SW-41 rotor facilitated the resolution of the 60 and 40S ribosome subunits and of the 80S monosomes. Polysomes sedimented to the bottom of the gradient, and the 4S tRNA peak is visible near the top (Fig. 9A). Treatment of viral polysomes with puromycin induced premature termination and release of nascent protein; with subsequent incubation, the ribosome subunits were dissociated and released the mRNA molecules. After this treatment, the 80S monosome and polysome peaks disappeared with a concomitant increase in material sedimenting in the ribosomal subunit peaks (Fig. 9A). If one dissociates polysomes with the puromycin treatment and sediments as described, the net acid-insoluble virus-specific RNA released from polysomes migrates between the 40 and 60S subunits (Fig. 9B). This is the position in the gradient where the viral 45S RNA would be expected to sediment. The material sedimenting at the bottom of the gradient is probably caused by incomplete dissociation of the viral polysomes.

DISCUSSION

Analysis of intracellular SLE virus-specific RNA by agarose gel electrophoresis demonstrates the presence of two types of RNA molecules in SLE-infected cells. These are the genome-sized 45S molecule and small 8 to 10S molecules. With one exception, all previous investigators of flavivirus intracellular RNAs used sucrose gradients as the means of analysis. These studies all report the presence of the 45S mole-

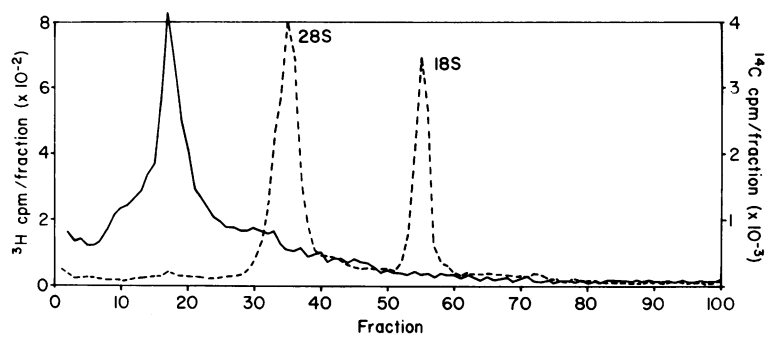

FIG. 8. *Electrophoresis of SLE virus polysome-associated RNA on denaturing agarose gels. [^{3}H]uridine-labeled SLE RNA* (——), *[^{14}C]uridine cellular rRNA* (- - -).

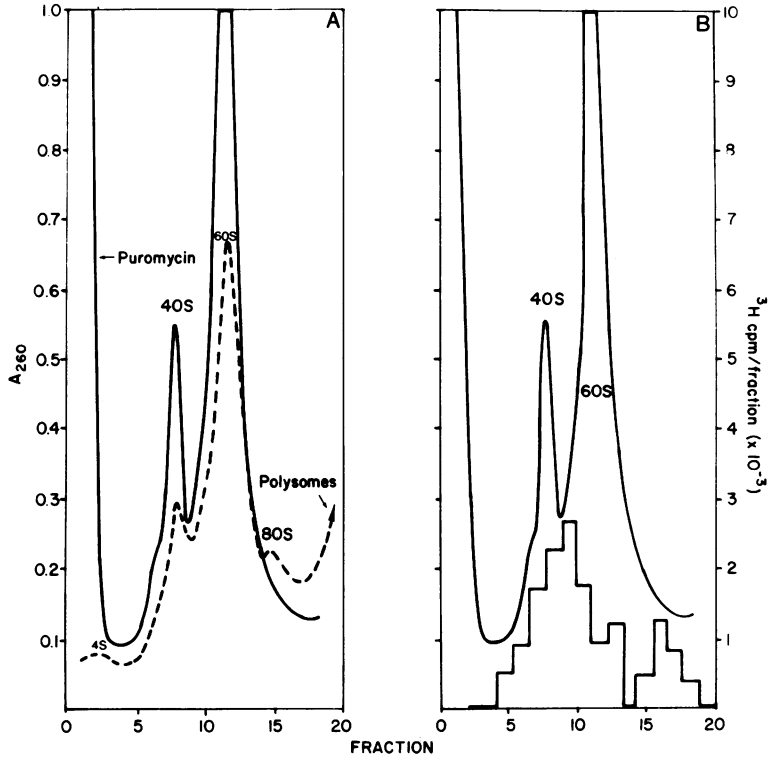

FIG. 9. *Sedimentation of polysomes, monosomes, ribosomal subunits, and SLE virus mRNA on 5 to 20% sucrose gradients in TKM buffer. (A) A_{260} optical density profile of components without puromycin treatment (- - -) and with KCl-puromycin treatment (——). (B) Distribution of ^3H-labeled SLE virus polysome-associated RNA released by puromycin treatment. (——) A_{260}; the histogram indicates the ^3H counts per minute in treated profile minus ^3H counts per minute in untreated profile.*

cules and a 20S RNase-resistant species (9, 22, 24, 27, 31). The 26S and 8 to 10S molecules are seen in some but not all of these studies. The exception, a publication by Harley et al. (10), used polyacrylamide gel electrophoresis, and although they did not find a 20S RNase-resistant species, they did find 45S and 26S RNA molecules similar to those seen in Sindbis virus-infected cells.

Careful scrutiny of the publications dealing with flavivirus RNA reveals that in all of the studies in which a high MOI was used, the 26S peak in the RNA profiles is prominent (16, 24, 27), and in those in which a low MOI was used the 26S peak is reduced or nonexistent (9, 22, 31, 32). It thus seems plausible that the single-stranded 26S RNA observed by some workers is defective interfering particle RNA. Based on in vitro studies, Darnell and Koprowski (7) suggested that flaviviruses do produce defective interfering particles.

The 20S molecule has been variously described as RNase resistant (9, 22, 24, 27, 31) and as a mixture of RNA molecules of varying RNase resistivity (9). Stollar et al. (22) treated the 20S peak recovered from a sucrose gradient with dimethyl sulfoxide and recentrifuged the treated RNA. The denatured RNA sedimented as virion 45S RNA. The authors concluded that the 20S molecule is a double-stranded 45S molecule. This species would then, of necessity, have a molecular weight of approximately 8×10^6. Our investigation failed to reveal any molecule larger than the 4×10^6-dalton 45S molecule (data not shown). We noted minor 20S peaks on SDS-sucrose gradients, but no corresponding peaks were observed on the agarose gels. These observations seem to indicate that the 20S molecule is a conformer of the 45S species, a possibility which cannot be excluded by the observations of Stollar et al. (22).

Our failure to find a double-stranded viral RNA in cells infected with SLE virus was unexpected since an RNase-resistant 20S species has previously been reported in flavivirus-infected cells (9, 22, 24, 26, 27). Boulton and Westaway (Acta Virol., in press) have recently reported that single-stranded 43S RNA is the only species of viral RNA present in cells infected with the flavivirus Kunjin.

The mechanism by which the replication of flavivirus 43S RNA is carried out is not well understood. Since replicative double-stranded forms of the genome are not present in the cell and replication of the positive strand probably occurs on a genome-sized negative-strand template that is a copy of the parental 43S RNA, some mechanism must exist to prevent double-stranded RNA from forming. Perhaps the nascent RNA is rapidly coated with nucleocapsid protein such that a true duplex molecule is not formed, as has been postulated for vesicular stomatitis virus by Huang and associates (11, 21).

Our analysis of polysomes in SLE virus-infected cells confirms that flaviviruses do not shut off host cell protein synthesis. As would be expected, SLE virus mRNA is sensitive to RNase and is released from polysomes by EDTA. The material containing viral RNA that migrates at 140S as a contaminant has not been identified but is thought to be nucleocapsid (19, 22, 26).

Our results agree with the report of Cleaves and Schlesinger (Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, S50, p. 287) that the 45S virus RNA is the only mRNA in flavivirus-infected cells. It has been recovered from polysomes free of contaminating nucleocapsids, is released from polysomes by EDTA and puromycin, and is sensitive to RNase. The ultimate criterion, its translatability in vitro, is currently being investigated and will be reported at a later date.

Thus, flaviviruses apparently do not replicate in the same manner as alphaviruses, in that the genome RNA serves as the only mRNA, nor in the same manner as picornaviruses, in that post-translational cleavage is not observed and the virus is capable of synthesizing all nine virus-specific polypeptides simultaneously after synchronized initiation (30). Apparently flavivirus mRNA has multiple internal initiation sites, which either are recognized by normal cell processes or require a virus polypeptide to alter the cell translation mechanism such that internal initiation sites are recognized.

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