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# Viral RNAs Associated with Ribosomes in Sindbis Virus-Infected HeLa Cells

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Virus specific RNA ribosome complexes were isolated by sucrose density gradient centrifugation of cytoplasmic extracts from HeLa cells infected at 42 C with an RNA$^+$ mutant (ts2) of Sindbis virus. Viral RNA-ribosome complexes were accumulated by infected cells treated with sodium fluoride and cycloheximide. The RNA-ribosome complexes were characterized by (i) their sensitivity to the action of ribonuclease or ethylenediaminetetraacetic acid, (ii) their density in cesium chloride gradients, and (iii) presence of host ribosomes and viral RNAs. The viral RNAs were isolated and characterized. The results showed that two species of single-stranded RNAs (a 28*s* and 18 to 15*s* species) were associated with the complexes. Base composition analysis of the viral RNAs indicated that both species had a higher adenine content than the 42*s* or 26*s* forms of viral RNAs. The RNAs associated with the ribosome complexes were virus specific since they annealed with denatured double-stranded RNAs from the infected cells. Little or no 42S RNA was associated with the RNA-ribosome complexes. The results suggest that the 28*s* and 18 to 15*s* forms of RNAs may represent viral messenger RNAs.

At present the precise mode of formation of viral proteins in arbovirus-infected cells is unknown. Partly, this is due to the lack of information concerning the viral messenger RNA in arbovirus-infected cells. The single-stranded viral RNA present in the virions is infectious (39, 46). This suggests that the virion RNA, as a whole or in part, serves as a messenger in the initiation of viral protein synthesis in vivo. However, there are no direct experimental data on the nature of arboviral messenger RNA. The present report is an attempt to identify the viral messenger RNAs in HeLa cells infected with Sindbis (SB) virus.

Cytoplasmic extracts of cells infected with arboviruses contain several virus-specific structures including (i) mature virions and nucleocapsids with sedimentation rates of 260*s* and 140*s*, respectively (16, 38); (ii) replication complexes (structures containing viral RNA polymerase and nascent viral RNAs) possessing a sedimentation rate of about 300*s* (41); (iii) a 65*s* structure (16, 38); and (iv) virus-specific polyribosomes. Thus, analysis of cytoplasmic extracts from infected cells on sucrose density gradients yields polyribosomes contaminated with other virus-specific structures. Mature virus or nucleocapsids are absent in cells infected with the ts2 mutant of SB virus since the mutant is unable to form nucleocapsids at 42 C (5, 48). Therefore, in the present study cytoplasmic extracts from HeLa cells infected with the ts2 mutant at 42 C were used to isolate and characterize the viral messengers. The probable contamination of replicative complexes in sucrose density gradient fractions containing viral polysomes was circumvented by the following experimental approach.

Sodium fluoride inhibits protein synthesis in mammalian cells (8, 9, 22, 30, 45). Recently Vesco and Colombo (45) observed that sodium fluoride disaggregates polyribosomes in HeLa cells and prevents the dissociation of monosomes into ribosomal subunits. They also observed that HeLa cell messenger RNAs were conserved during the sodium fluoride-induced disaggregation of polyribosomes and consequently the messenger RNA molecules released from polysomes remained associated with the monosomes. The RNA-monosome complexes accumulated in HeLa cells in the presence of sodium fluoride. The above findings prompted us to search for viral messenger RNA-ribosome complexes in infected HeLa cells incubated with sodium fluoride. Such complexes should have sedimentation rates only slightly higher than the monosomes, since Vesco and Colombo (45) established that HeLa

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cell messenger RNA-ribosome complexes possess sedimentation rates of about 95 to 100s. Consequently, sucrose density gradient fractions containing such complexes will be free of virus-specific replication complexes. Cycloheximide, an inhibitor of protein synthesis, was also used in conjunction with sodium fluoride since the former inhibits the translational movement of ribosomes along the messenger RNAs (47) and this prevents the nonspecific breakdown of polyribosomes.

## MATERIALS AND METHODS

**Cells and virus.** Primary cultures of chicken embryo (CE) cells were prepared and grown in Eagle medium (39). The strain of HeLa cells used was obtained from T. W. Schaffer, Schering Corp., Bloomfield, N.J. The HeLa cells were grown as monolayers using Eagle medium containing 10% fetal calf serum. Infectious virus was assayed in CE cells by methods described earlier (39).

The source and preparation of SB virus, HR strain, or the temperature-sensitive mutant ts2 and the methods used to infect cells and to label viral RNA were identical to those described previously (41, 48). Experiments in which cell cultures were incubated at 27 or 42 C were performed in air incubators in the presence of a modified Eagle medium. The details of the above experimental procedures were described earlier (37). Stock preparations of ts2 mutant were obtained by growing the virus in CE cells at 27 C. Assays for infectious virus in CE cells at 27 and 42 C indicated that the virus stocks contained less than 1% revertants.

**Chemicals and isotopes.** Actinomycin D was obtained as a gift from Merck, Sharp and Dohme (West Point, Pa.), and was used at a concentration of 5 μg/ml to inhibit synthesis of cellular RNA. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was used at 100 μg/ml. Isotopically labeled amino acids and uridine were purchased from Nuclear Chicago Corp. (Des Plaines, Ill.). The specific activities of tritiated leucine and uridine were >20,000 mCi/mmol and >30,000 mCi/mmol, respectively. $^{14}$C-uridine (specific activity >50 mCi/mmole), $^{14}$C-amino acid mixture (specific activity, 45 mCi/matom) and $^{32}$P as phosphoric acid (specific activity, 50 to 100 mCi/mmol phosphorus) was purchased from New England Nuclear Corp. (Boston, Mass.).

**Preparation of cytoplasmic extract.** Monolayers of cells were washed with ice-cold phosphate-buffered saline, scraped from the dishes, and centrifuged at 3,000 rpm at 4 C. The cell pellet was suspended in RSB (0.01 M tris[hydroxymethyl]-aminomethane [Tris], 0.01 M NaCl, 0.0015 M $MgCl_2$, pH 7.4) and allowed to swell at 4 C for 10 min. The cells were ruptured by 10 complete strokes with a tight-fitting homogenizer. The homogenate was centrifuged at 5,000 rpm for 5 min at 4 C. The supernatant fluid was incubated with sodium deoxycholate (0.5%) and layered on 16 ml of a 15 to 30% sucrose density gradient in RSB. The gradients were centrifuged in an SW27.3 rotor (Spinco) at 4 C. Fractions were collected, and the acid-insoluble radioactivity in the fractions was assayed. Ribosomes (74s) from uninfected cells were used as standards for determining sedimentation rates.

**Centrifugation in cesium chloride gradients.** Isopycnic analysis of the cytoplasmic extracts from infected and uninfected cells was performed by methods similar to those described by Huang and Baltimore (24). The cytoplasmic extract was prepared and centrifuged at 4 C overnight on a 15 to 30% linear sucrose density gradient to separate polyribosomes, ribosomes, and ribosomal subunits. The sedimentation pattern of the radioactivity in the gradient was determined by assaying the amount of trichloroacetic acid-precipitable radioactivity contained in the fractions. Fractions representing polyribosomes, ribosomes, or ribosomal subunits were pooled separately and fixed with 6% glutaraldehyde (pH 7.4) at 4 C. The samples were layered immediately on a 33 to 55% CsCl gradient in RSB containing 0.8% Brij-58 (Atlas Chemical Co.). Cellulose nitrate centrifuge tubes were used since polyallomer tubes are known to produce artifacts (24). The samples were centrifuged at 45,000 rpm for 24 h. Fractions were collected and assayed for acid-precipitable radioactivity. The refractive index and the density of the fractions were determined by methods similar to those used by Huang and Baltimore (24).

**Isolation of RNA.** Viral or cellular RNAs from cells were isolated by a modified phenol method. The cell pellet was suspended in $10^{-2}$ M ethylenediaminetetraacetic acid-sodium salt (EDTA) at pH 7.4. Samples of stock solutions of Pronase (Calbiochem) were added to the cell suspension to make a final concentration of 500 μg/ml of Pronase. Stock solutions of Pronase containing 2 mg/ml were prepared by the method of Stern (42). The cell suspension was incubated at 35 C for 15 min, and then sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5%. The samples were incubated for an additional 15 min at 35 C. An equal volume of redistilled phenol (saturated previously with $10^{-2}$ M EDTA, pH 7.4) was added to each sample and mixed by constant stirring at 25 C. The aqueous and phenol layers were separated by centrifugation at 10,000 rpm for 1 min. The lower layer consisting of phenol was carefully removed. An equal volume of phenol was added to the aqueous layer, and the extraction procedure was repeated twice. The aqueous and the interphase layers remaining at the end of the extraction procedure were mixed with 2.5 volumes with cold 95% ethanol. Unlabeled yeast RNA served as a carrier during the above step. The yeast RNA used was purified from commercial yeast RNA (Calbiochem) by extraction with phenol and precipitation with alcohol. The nucleic acid was precipitated with alcohol by incubation at −20 C for at least 2.5 h. The precipitate was collected by centrifugation at

10,000 rpm for 10 min at 4 C and dissolved in a buffer consisting of 0.05 M Tris (pH 7.4) and 0.1 M NaCl. The DNA present in such preparations was removed by the following procedure. Purified DNase (RNase-free) and magnesium chloride were added to the samples to a final concentration of 100 μg/ml and $10^{-2}$ M, respectively. Then the samples were incubated at 37 C for 20 min. The DNAse present in the samples was removed by extraction with phenol as described earlier. The RNA was precipitated with ethanol and dissolved in a buffer consisting of 0.05 M Tris (pH 7.4), 0.1 M NaCl, and 0.001 M EDTA (TES). In some experiments the viral RNAs were precipitated by 2 M lithium chloride according to methods described earlier (41).

**Isolation of double-stranded form of SB virus RNA.** SB virus-specific double-stranded RNA was prepared and purified as follows. Total RNA consisting of cellular and virus-specific RNAs were isolated from infected CE cells. The procedures for the isolation of the total RNA free of cellular DNA were identical to those described in the previous section. Lithium chloride (2 M) was used to precipitate the single-stranded and partially double-stranded forms of RNA present in the total RNA. Under the above conditions, the double-stranded form of viral RNA and transfer RNA remain soluble (41). The double-stranded RNA present in the salt-soluble fraction was separated from the transfer RNA by chromatography on cellulose (Whatman CF 11) columns as described by Billeter et al. (2). The RNA eluting from the cellulose column in the presence of buffer was recovered and purified further by a second cycle of chromatography on the cellulose column. Approximately 10 μg of double-stranded RNA was obtained from $5 \times 10^8$ infected CE cells.

**Hybridization of SB RNA.** The procedures used for hybridization of SB viral RNA will be described in detail later (Segal and Sreevalsan, manuscript in preparation). In brief, the procedure used was as follows. Samples of RNAs in 0.1 × SCC (SCC = 0.15 M NaCl, 0.015 M sodium citrate) were placed in glass ampoules (Bellco Glass Co., N.J.) and consisted of labeled single-stranded RNAs with or without unlabeled double-stranded RNA, or a mixture of labeled and unlabeled single-stranded RNA with unlabeled double-stranded RNA. The final volume of samples was 50 μliters. Denaturation of RNA was accomplished by incubating the ampoules in a boiling-water bath for 5 min. The ampoules were then chilled in an ice bath, and 50 μliters of 12 × SCC was added. Annealing was performed by placing them in a water bath at 78 C for 4 h. At the end of annealing the samples were chilled, and 0.5 ml of sterile water was added to each sample. The samples were incubated with 2 μg of ribonuclease per ml at 37 C for 15 min. The samples were precipitated with an equal volume of 10% trichloroacetic acid, filtered, and counted.

**Analysis of viral RNA.** The analysis of RNA was performed by centrifugation on sucrose density gradients or by electrophoresis on polyacrylamide gels. The methods involving the use of sucrose density gradients were reported previously (37, 38). RNA was analyzed by electrophoresis on polyacrylamide gels according to the method of Peacock and Dingman (32). Polyacrylamide gels (2.2%) containing 0.5% agarose were prepared. Gels (80 by 6 mm) were prepared in Plexiglas tubing. Electrophoresis was carried out at room temperature using a buffer containing 0.108% Tris (pH 8.3), 0.093% EDTA, 0.55% boric acid, and 0.3% SDS. After electrophoresis, the gels were fixed with 10% trichloroacetic acid and then sliced into 1.3-mm segments with a slicing device designed by Chrambach (7). Each slice was placed in a scintillation vial and incubated overnight at 55 C with 0.05 ml of 33% hydrogen peroxide. The samples were counted in a Packard scintillation counter, model 3375. The scintillation fluid used consisted of 1 liter of toluene, 1.5 liters of Triton X-100, 4 g of 2,5-diphenyloxazole, and 0.05 g of 1,4-*bis*-2-(5-phenyloxazolyl)-benzene. Samples containing $^3H$ and $^{14}C$ isotopes were assayed for radioactivity with the discriminator setting which allowed only 9.1% of the $^{14}C$ label to appear in the $^3H$ channel. Appropriate corrections for quenching were made to estimate the $^3H$ or $^{14}C$ radioactivity in the samples.

**Assay for radioactivity.** The methods used were described previously (37).

**Base composition analysis of viral RNA.** This was performed by the method of Katz and Coomb (26). $^{32}P$-labeled viral RNAs were used. Three separate determinations were carried out for each sample, and the average values are presented.

## RESULTS

**Growth characteristics of SB virus in HeLa cells.** Buckley (4) studied the replication of arboviruses in several cell lines. Cells from different hosts were found to vary in their susceptibility to these viruses. The S3 strain of HeLa cells is nonpermissive for SB virus (Rosemond and Sreevalsan, unpublished data). However, another strain of HeLa cells known as HeLa R (Grand Island Biological Co., New York) was found to support the replication of SB virus. Single-step growth cycles of the HR and ts2 mutant of SB virus at 37 and 27 C, respectively, were carried out in the above cells. The results of such an experiment are presented in Fig. 1. The results indicate that infectious virus is synthesized in HeLa cells infected with HR or ts2 strain of SB virus. The exponential rise of virus in HR-infected cultures was between the 4th and 6th h, whereas in ts2-infected cells it occurred between the 6th and 8th h. The ts2-infected cultures were incubated at 27 C and, at this temperature, the biosynthesis of virus is usually slower than at 37 C (5). Maximal yields

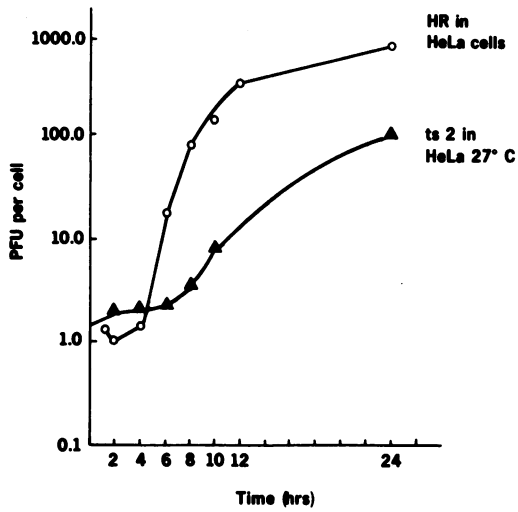


FIG. 1. *Single-step growth cycles of HR and ts2 strains of Sindbis virus in HeLa R cells. Twenty-four-hour-old monolayers of HeLa cells (2.0 × 10⁶ cells per culture) were infected with ts2 or HR strains of Sindbis virus at 25 C for 30 min. The cultures were then washed three times with phosphate-buffered saline. A 3-ml amount of Tricine medium or minimal essential medium containing fetal calf serum were added to cultures infected with ts2 or HR strain, respectively. The cultures infected with ts2 were incubated at 27 C; the cultures infected with HR were incubated at 37 C. Duplicate cultures were harvested at times indicated. Samples were frozen and thawed three times before assaying for the virus. Assays for infectious virus of ts2 strain were performed at 27 C; those of HR strain were performed at 37 C. Symbols: ○, HR strain; ▲, ts2 strain.*

of virus were obtained at about 36 h after addition of virus to cultures. HeLa cells produced about 1,500 PFU of HR virus or 100 to 150 plaque-forming units of ts2 virus per cell. These results indicate that the strain of HeLa cells used can support the multiplication of SB virus.

The ts2 strain of SB virus is an RNA$^+$ mutant, i.e., viral RNA and proteins are synthesized in CE cells at both permissive (27 C) and nonpermissive (42 C) temperatures (5). Preliminary experiments indicated that the rate of synthesis of viral RNA or protein was similar in HeLa cells infected at 42 C with either HR or ts2. Experiments were performed to determine whether all the known forms of SB viral RNAs were synthesized at 42 C in HeLa cells infected with ts2. The results (Fig. 2) were obtained when the salt-insoluble forms of viral RNAs (single-stranded viral RNAs and replicative intermediate) were analyzed by centrifugation on sucrose density gradient or electrophoresis on polyacrylamide gels. Based on the sedimentation rates of the $^{14}$C-uridine-labeled ribosomal RNAs from HeLa cells, the two major species of viral RNAs ($^3$H-uridine-labeled) sedimented in the gradient at rates of 42*s* and 26*s*, respectively (Fig. 2a). The results also indicate the presence of a minor 15*s* species of viral RNA. Trace amounts of 38*s* and 32*s* RNA species were also observed. The double-stranded form of RNA was absent in the sample of viral RNAs analyzed, since it was removed during the step involving precipitation with lithium chloride. The electropherogram obtained subsequent to analysis of a mixture of $^3$H-uridine-labeled viral and $^{14}$C-uridine-labeled ribosomal RNAs on polyacrylamide gels is shown in Fig. 2b. Radioactivity due to $^3$H is distributed in the gel in five peaks (1 to 5). Some of the radioactivity due to $^3$H remains at the origin of the gel. The radioactivity due to $^{14}$C representing the 28*s* and 18*s* ribosomal RNA species is distributed in two peaks. Thus five species of single-stranded viral RNA forms were detected. These results are in agreement with those reported by others (6, 13, 29). Previous results (not shown here) have indicated that peaks 1 and 4 correspond to the 42*s* and 26*s* forms of SB viral RNAs. The radioactivity found at the origin of the gel may represent the replicative intermediate (RI) form of RNA since it has been shown that purified preparations of polio or SB virus RI do not enter the gel during electrophoresis under similar conditions (31; Sreevalsan et al., unpublished data). The peaks represented as 2 and 3 constitute only a small fraction of the total single-stranded viral RNA forms. The predominant species of single-stranded viral RNAs are peaks 1, 4, and 5. The present results differ from those of Levin and Friedman (29) wherein peak 5 species of RNA was not found to be a major species. The above authors failed to remark on the presence of the peak 5 species of RNA, even though their results indicated the presence of such a species. Thus, the ts2 strain of BS virus induces all the known forms of single-stranded viral RNAs in HeLa cells at 42 C.

The primary purpose in using ts2 mutant of SB virus in the present study is the possibility that cytoplasmic extracts free of viral nucleocapsids can be obtained from cells infected at 42 C. The results (Fig. 3) indicate that HeLa cells infected with ts2 virus at 42 C contain little or no nucleocapsids. However, a similar cytoplasmic extract from cells incubated at 27 C (permissive temperature) contain radioactivity localized mainly in two regions of the gradient: peaks at 140*s* and 65*s* (Fig. 3b). The radioactivity remaining at the top of the gradients may represent free viral RNAs. Structures similar to the 140*s* and 65*s* have been reported previously

by others (1, 16, 38). The 140s structure represents viral nucleocapsids.

The results obtained so far can be summarized as follows: (i) The growth characteristics of HR and ts2 strains of SB virus in HeLa cells are similar. (ii) The ts2 strain of SB virus induces all known forms of single-stranded viral RNAs in HeLa cells at 42 C. (iii) Viral nucleocapsids are not formed during infection of HeLa cells with ts2 virus at 42 C.

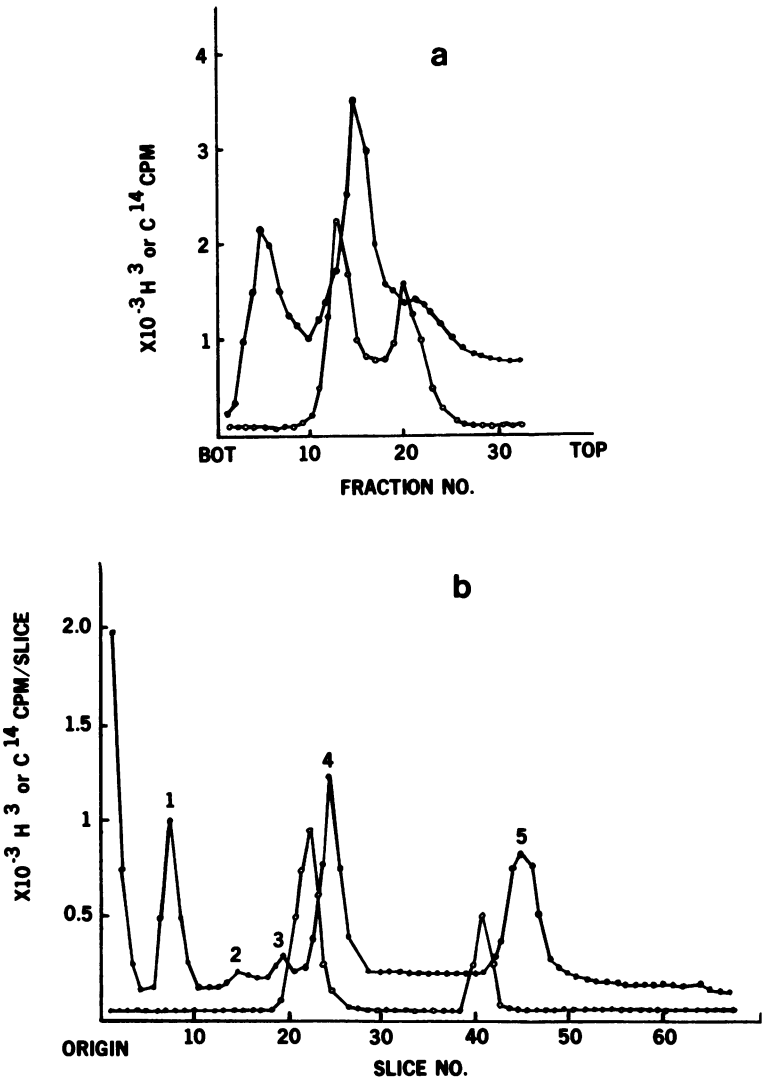


FIG. 2. *Analysis of viral RNAs found in infected HeLa cells. Monolayers of HeLa cells were incubated with medium containing actinomycin D (5 μg/ml). One hour later the cells were infected with ts2 virus and then incubated at 42 C. Four hours later $^3$H-uridine (200 μC/culture) was added to the cultures. Incorporation was allowed to occur for 1 h, at the end of which time the cells were harvested for the isolation of RNA. The RNA was fractionated by precipitation with 2 M lithium chloride. A sample of the salt-precipitated RNA (free of the double-stranded form of viral RNA) was mixed with a sample of $^{14}$C-uridine-labeled ribosomal RNA from uninfected HeLa cells. A sample of the above mixture was analyzed by sucrose density gradient centrifugation or by polyacrylamide gel electrophoresis. a, Pattern of the distribution of radioactivity contained in fractions obtained after the RNA mixture was centrifuged on a 16-ml gradient of 6 to 30% sucrose in TES. Centrifugation was performed with a 27.3 rotor at a speed of 22,500 rpm for 14 h at 4 C. b, Electrophoresis of 50 μliters of the RNA mixture on 2.2% polyacrylamide-agarose column was carried out at 6 mA/gel for 2 h at 25 C. Symbols: ○, $^{14}$C; ●, $^3$H.*

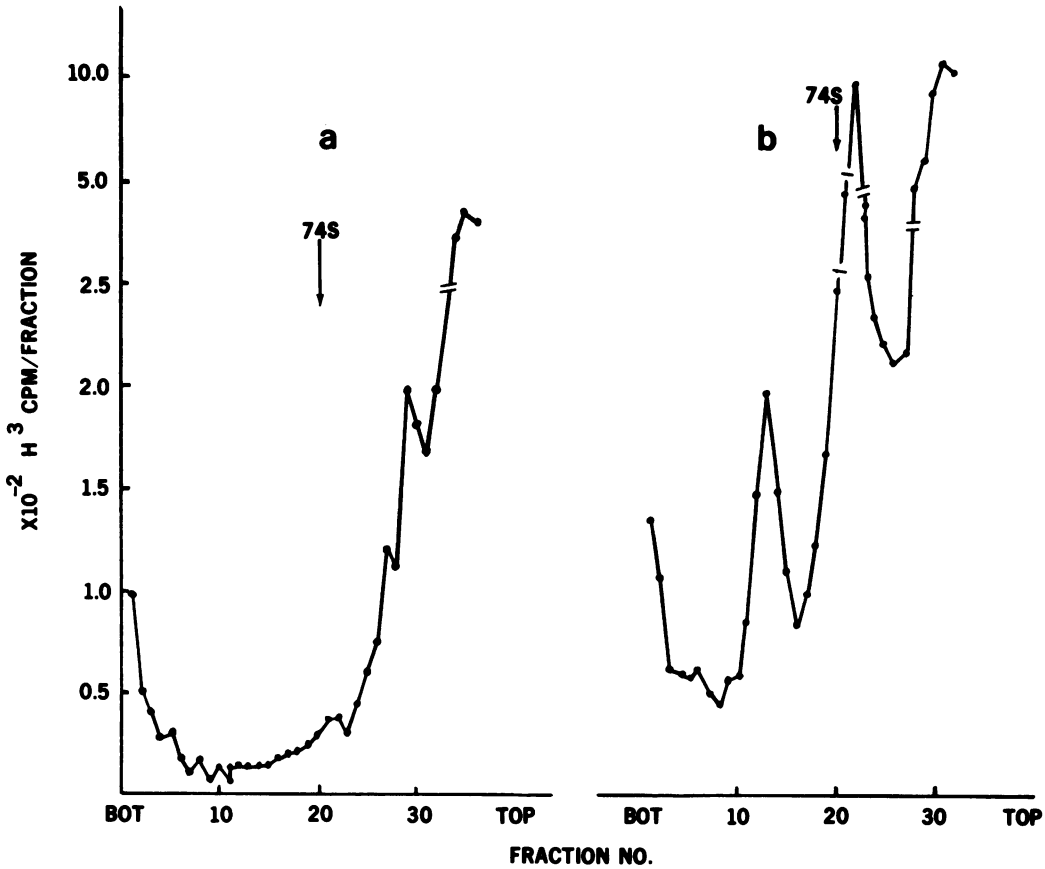


FIG. 3. *Sucrose density gradient analysis of cytoplasmic extracts from HeLa cells infected with ts2 virus. Monolayers of HeLa cells were infected with ts2 virus and incubated at 27 or 42 C in the presence of actinomycin D (5 μg/ml). Tritiated uridine (25 μCi/culture) were added to the cultures at the 4th h after addition of virus to culture. Incorporation of the radioactivity was allowed to occur for 60 min. Cytoplasmic extracts were prepared from the infected cells and used for analysis on 16-ml gradients of 7.5 to 30% sucrose in RSB. Centrifugation was performed with a 27.3 Spinco rotor at a speed of 12,000 rpm for 14 h at 4 C. Single ribosomes were assigned a value of 74s under the above conditions, and the arrow in the figure represents the position at which they sediment in the gradient. Cytoplasmic extract from cultures incubated at (a) 42 C and (b) 27 C.*

Thus cytoplasmic extract from HeLa cells infected with ts2 virus at 42 C can be used for the isolation of SB virus-specific messenger RNAs.

**Analysis of cytoplasmic extracts from uninfected and infected HeLa cells.** The experiments described in the previous sections indicated the suitability of using HeLa cells infected with ts2 virus at 42 C as a system for the isolation of virus-specific polyribosomes. Therefore, the following experiment was performed to ascertain whether viral RNA-ribosome complexes can be isolated from HeLa cells infected with ts2 at 42 C.

Several monolayers of HeLa cells ($8.0 \times 10^6$ cells per culture) were incubated with medium containing $^3$H-uridine (2 μCi/culture) for 24 h to label the cellular ribosomes. The cultures were washed several times and then incubated for an additional 24 h with medium containing no radiolabel to deplete the intracellular pool of $^3$H-uridine. Some of the cultures were infected with the ts2 mutant. The rest of the cultures were mock infected with phosphate-buffered saline. The cultures were incubated at 27 C for 4 h with medium containing 5 μg of actinomycin D per ml. Then the cultures were washed and incubated at 42 C. One hour later, $^{14}$C-uridine (50 μCi/culture) was added to the cultures. Incorporation was allowed to occur for 30 min, at the end of which time cycloheximide (100 μg/ml) was added to the cultures. Incubation was continued for 10 min, and then sodium fluoride was added to

obtain 0.015 M. Twenty minutes later the cells were harvested and cytoplasmic extracts were prepared.

The results presented in Fig. 4 illustrate the sedimentation pattern of the radioactivity found in the cytoplasmic extracts of uninfected and infected cells. The sedimentation pattern seen in Fig. 4a shows the distribution of the $^3$H-uridine-labeled ribosomes in uninfected HeLa cells in the presence of cycloheximide and NaF. The data indicate the presence of structures sedimenting faster than the 74*s* monoribosomes (the peak of radioactivity in fraction 27 represents 74*s*). These structures probably represent polyribosomes (45). However, they contained little or no radioactivity due to $^{14}$C-uridine. This was not surprising since the cultures were incubated with actinomycin D before the addition of $^{14}$C-uridine. The localization of the radioactivity due to $^{14}$C and $^3$H-uridine in the cytoplasmic extract from the infected cells is illustrated in Fig. 4b. The results indicate that the sedimentation pattern of host polyribosomes ($^3$H-uridine label) is altered as a result of the infection. Structures containing host ribosomes sedimenting at rates faster than the 74*s* are also present in the cytoplasmic extract of the infected cells. There are relatively smaller amounts of these structures in the infected cells when compared with the uninfected cells. However, unlike the results obtained from uninfected cultures, structures containing $^{14}$C-uridine are distributed throughout the gradient. The radioactivity due to $^{14}$C-uridine is contained mainly in a peak localized slightly below the 74*s* peak of monosomes. This is seen in Fig. 4b wherein the radioactivity due to $^3$H-uridine is localized as a peak in fraction 26 (74*s*) while the radioactivity due to $^{14}$C-uridine is found as a peak in fraction 23 (100*s* to 90*s*). In this context, it should be noted that $^{14}$C-uridine gets incorporated only into virus-specific structures since cells incubated with actinomycin D alone did not contain such structures (compare Fig. 4a). The 100 to 90*s* virus-specific structure seen in Fig. 4b may possibly be similar to the 95*s* particles observed by Vesco and Colombo (45) in uninfected HeLa cells incubated with NaF. The virus-specific structures sedimenting faster than the 74*s* may represent viral nucleocapsids, viral replicative complexes, polyribosomes, random complexes of RNA with proteins, or complexes of messenger RNA with monosomes. The presence of radio-labeled viral nucleocapsids in the cytoplasmic extract of infected cells can be ruled out on the basis of the results presented in Fig. 3. EDTA or RNase was used to distinguish the other possibilities mentioned above. EDTA is known to quantitatively dissociate messenger RNAs from polyribosomes and monosomes (21). However, under the above conditions, viral replicative complexes remain unaffected (21). Incubation of cytoplasmic extract with EDTA does not change the sedimentation rate of random RNA-protein complexes (20). Therefore, the only structures unaffected by incubation of cytoplasmic extracts from infected cells with EDTA are viral replicative complexes and random RNA-protein complexes. Also, polyribosomes are extremely sensitive to the action of RNase. Thus, if the virus-specific structures sedimenting at rates faster than 74*s* (Fig. 4b) are indeed polyribosomes and messenger RNA-monosome complexes, they should be dissociated by EDTA or RNase. The results presented in Fig. 4c and 4d represent experiments wherein samples of the cytoplasmic extracts from infected cells as used in Fig. 4b were incubated with $10^{-2}$ M EDTA or 10 $\mu$g of RNase per ml for 10 min at 4 C and then subjected to centrifugation on gradients of sucrose. Incubation of cytoplasmic extracts with EDTA decreased the amount of radioactivity ($^3$H or $^{14}$C) sedimenting at rates greater than 74*s* (Fig. 4c). Incubation of the cytoplasmic extract with RNase resulted in a similar decrease in the amount of the faster-sedimenting structures. Comparison of the above results with those presented in Fig. 4b (no EDTA or RNase) indicates that the majority of the structures from cytoplasmic extracts of infected cells sedimenting at rates faster than 74*s* are possibly polyribosomes or messenger RNA-monosome complexes. The EDTA or RNase-resistant structures which are seen in fractions 1 to 10 (Fig. 4c and 4d) may possibly represent viral replicative complexes. The viral RNA-ribosome peak (100 to 90*s*) as seen in Fig. 4b is sensitive to EDTA since no corresponding peak is observed in Fig. 4c. These results also rule out the possibility that the 100 to 90*s* structures found in cytoplasmic extracts from infected cells may represent random protein-RNA complexes.

**Characterization of messenger RNA complexes by centrifugation on gradients of CsCl.** Studies on ribonucleoprotein complexes contained in subcellular materials have been performed by techniques involving centrifugation on CsCl gradients (24, 33, 36). Thus Huang and Baltimore used the virus-specific polyribosomes and ribosome-viral RNA complexes contained in cytoplasmic extracts of HeLa cells infected with polio virus (24). The buoyant density of pure RNA in CsCl is about 1.90 (3) and that of most proteins is about 1.30 g/ml (23). Ribosome-RNA complexes may possess buoyant densities varying from 1.30 to 1.90 g/ml (24). Preliminary

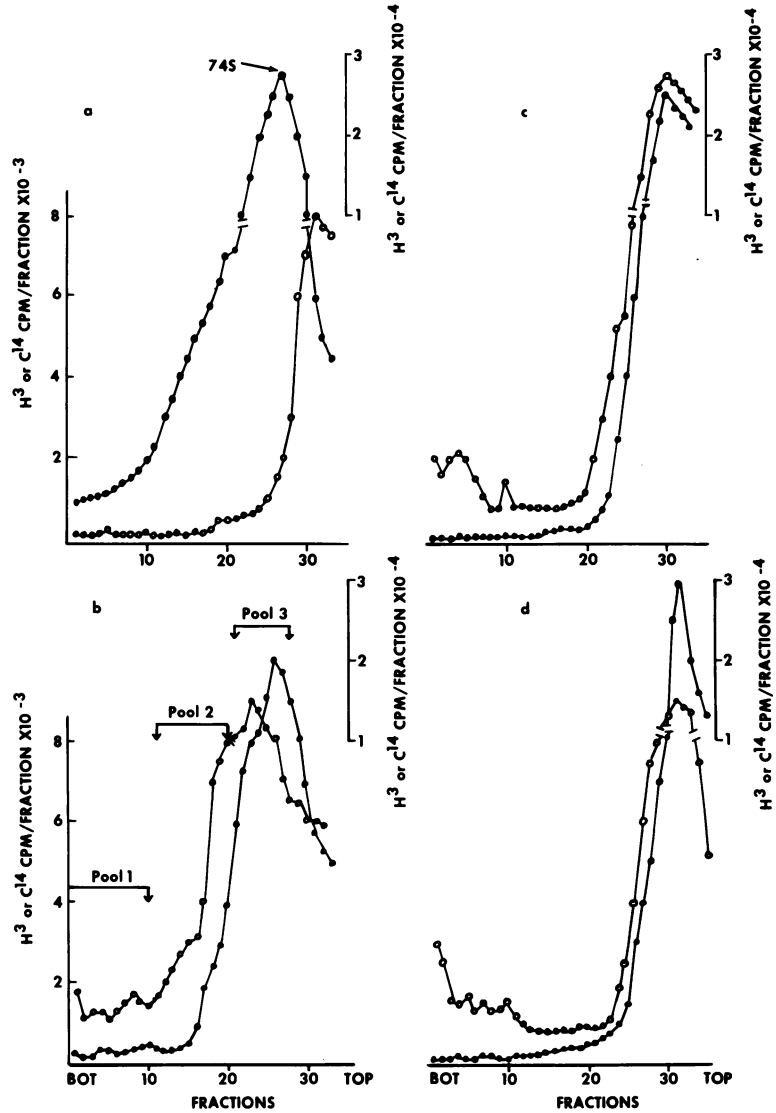


FIG. 4. *Analysis of cytoplasmic extracts from uninfected and infected HeLa cells. Monolayers of HeLa cells were incubated for 24 h with medium containing 2 μCi of ³H-uridine per ml. At the end of the incubation period, cultures were washed several times and incubated with medium free of isotope for 24 h. Monolayers were then infected with ts2 strain of SB virus or mock-infected with phosphate-buffered saline. Both the uninfected and infected cultures were incubated at 27 C with medium containing actinomycin D (5 μg/ml). The cultures were washed with prewarmed (42 C) medium at the 4th h after addition of virus to cultures and then incubated at 42 C. Incorporation was allowed to occur for 30 min, at the end of which cycloheximide (100 μg/ml) containing medium was added to all cultures. Incubation at 42 C was continued for an additional 10 min, at the end of which all cultures received enough sodium fluoride to obtain a final concentration of 15 mM. The cultures were incubated at 42 C for 20 min. Cytoplasmic extracts from the cells were prepared and analyzed by centrifugation on 16-ml gradients of 15 to 30% sucrose in RSB. Centrifugation was performed with a 27.3 rotor at a speed of 24,000 rpm for 2 h at 4 C. a, Uninfected cells; b, infected cells. Samples of the cytoplasmic extract from infected cells were incubated with either $10^{-2}$ M EDTA (c) or 10 μg/ml of RNAse (d) at 4 C for 10 min. Symbols: ○, ¹⁴C; ●, ³H. The pools 1, 2, and 3 in Fig. 4b represent the fractions from the gradient which were used for the isolation and analysis of the RNAs in Fig. 6 and 7.*

experiments indicated that the 74, 60, and 40s ribosomal structures possessed buoyant densities of 1.53, 1.57, and 1.48 g/ml, respectively. These results are in agreement with those reported by Huang and Baltimore (24).

The buoyant densities of SB virus-specific structures were then determined. The plan and the details of the experiment were identical to those described in the previous section except in the types of radioactive precursors used for labeling the host and virus-specific structures. HeLa cells were incubated with $^{14}$C-uridine (5 $\mu$Ci/culture) for 24 h to label the ribosomes and then infected with the ts2 strain of SB virus. Tritiated uridine (200 $\mu$Ci/culture) was added to the cultures after infection and incubation with medium containing actinomycin D under conditions identical to those described in the previous section. Cycloheximide and NaF were used in a similar fashion to that described earlier. Cytoplasmic extracts were obtained and centrifuged on sucrose density gradients. The samples were centrifuged on sucrose density gradients for a longer time in order to obtain a better resolution of the region around 74s. The sedimentation pattern obtained from such a centrifugation is shown in Fig. 5a. It can be seen from the results that host ribosomal structures sedimented mainly in three peaks. The faster-sedimenting structures represent 74s, whereas the slower-sedimenting structures are 60s and 40s ribosomal subunits. The presence of $^{14}$C- and $^{3}$H-uridine label in the faster-sedimenting structures indicates the presence of structures containing host ribosomes and virus-specific RNAs. The distribution of radioactivity due to tritiated uridine was distinct from that due to $^{14}$C-uridine-containing host ribosomes. There exists a broad peak of tritium label in the sucrose density gradient which sediments at rates faster than 74s (Fig. 5a). This corresponds to the 100 to 90s peak observed in Fig. 4b. The buoyant densities of these subcellular structures were determined by centrifugation on CsCl. Fractions from the area b and c (Fig. 5a) were pooled and used for centrifugation on CsCl gradients. The results are presented in Fig. 5b and c. The distribution of the $^{14}$C-uridine label due to the host ribosomes in CsCl indicates that 75% of the total radioactivity is localized in one peak with a density of 1.51 g/ml (Fig. 5b and c). However, the tritium-labeled viral RNA distributed heterogeneously in peaks with densities of 1.51, 1.48, and 1.44 (Fig. 5b and c). Incubation of the fractions with RNase or EDTA before fixation and centrifugation in CsCl resulted in the total loss of tritium label from the above peaks of radioactivity (results not presented). Huang and Baltimore (24) reported that heterogeneous messenger RNA molecules associated with monosomes would have sedimentation rates varying from 80 to 150s and buoyant densities ranging from 1.47 to 1.52 g/ml. Therefore the radioactivity present in the structures with densities of 1.51 and 1.48 may represent viral messenger RNAs complexed with single ribosomes. This conclusion is substantiated by the following observations. (i) The presence of $^{14}$C-uridine-labeled ribosomes in the structures possessing densities of 1.48 and 1.51 g/ml; (ii) release of viral RNA ($^{3}$H radioactivity) in structures isolated under identical conditions from uninfected $^{14}$C-uridine-labeled HeLa cells. The presence of glutaraldehyde in fractions from the top part of the CsCl gradients interfered in the determination of their densities. Therefore no meaningful comment can be made on the minor peak of radioactivity due to $^{3}$H-uridine at a density of 1.44 g/ml.

**Isolation and characterization of RNAs from viral RNA-monosome complexes.** The results presented in the previous sections indicate that the cytoplasmic extract from infected HeLa cells contains virus-specific structures possessing properties of messenger RNA-ribosome complexes. The next step was to isolate and characterize the viral RNAs associated with the above structures. Appropriate fractions as described in Fig. 4b were pooled and used for isolating the RNAs.

The sedimentation rates of RNAs, obtained from the fractions originating from the various regions of the sucrose gradient (Fig. 4b), are shown in Fig. 6. The radioactivity due to the host RNAs ($^{3}$H-uridine-labeled) was localized mainly in two distinct peaks with sedimentation rates of 28 and 18s. However, the distribution of the radioactivity due to the $^{14}$C-labeled viral RNAs in the sucrose gradients was quite dissimilar to that of the $^{3}$H-labeled ribosomal RNAs. Heterogeneous species of viral RNAs were obtained from the virus-specific structures. The types of viral RNAs found in the various fractions differed depending on the region of sucrose density gradient (Fig. 4b) from which they originated. The rapidly sedimenting virus-specific structures (Fractions 1 to 10 and 11 to 20, Fig. 4b) contained viral RNAs with heterogeneous sedimentation rates (Fig. 6a and b). Partially or fully double-stranded RNA species are also present in the above population of molecules, since RNase-resistant RNA can be isolated from the above fractions (results not presented). However, the majority of the viral RNAs isolated from the structures present in fractions 21 to 28 (Fig. 4b) consisted of only two classes (Fig. 6c).

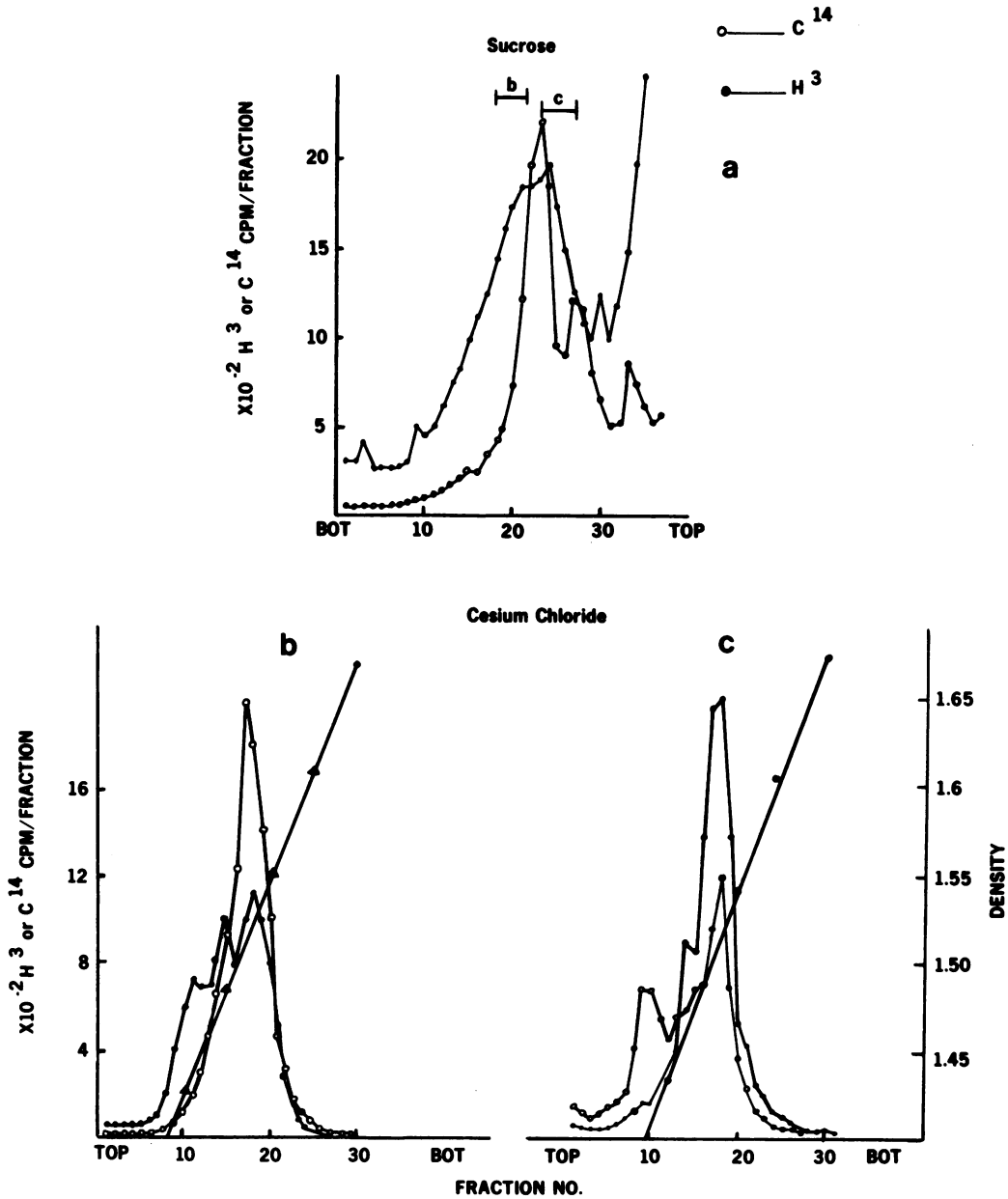


FIG. 5. *Analysis of cytoplasmic structures by centrifugation on gradients of cesium chloride. Monolayers of HeLa cells were infected with ts2 strain of SB virus under conditions similar to those described in the legend for Fig. 4. However,* $^{14}C$*-uridine was used for labeling the ribosomes, whereas* $^{3}H$*-uridine was used to label the virus-specific RNAs. Cytoplasmic extract was prepared and used for centrifugation on sucrose density gradient. Centrifugation was on a 16-ml gradient of 15 to 30% sucrose in RSB at 12,500 rpm for 14 h at 4 C in an SW27.3 rotor. Fractions were collected and assayed for radioactivity. a, Sucrose density gradient pattern of the cytoplasmic extract. Horizontal bars b and c in (a) indicate the fractions which were pooled and used for centrifugation on CsCl. b, CsCl centrifugation of the fractions from the sucrose density gradient sedimenting faster than 74s. c, CsCl centrifugation of the fractions from the sucrose density gradient sedimenting slower than 74s. Symbols: ▲, density; ○,* $^{14}C$*; ●,* $^{3}H$.

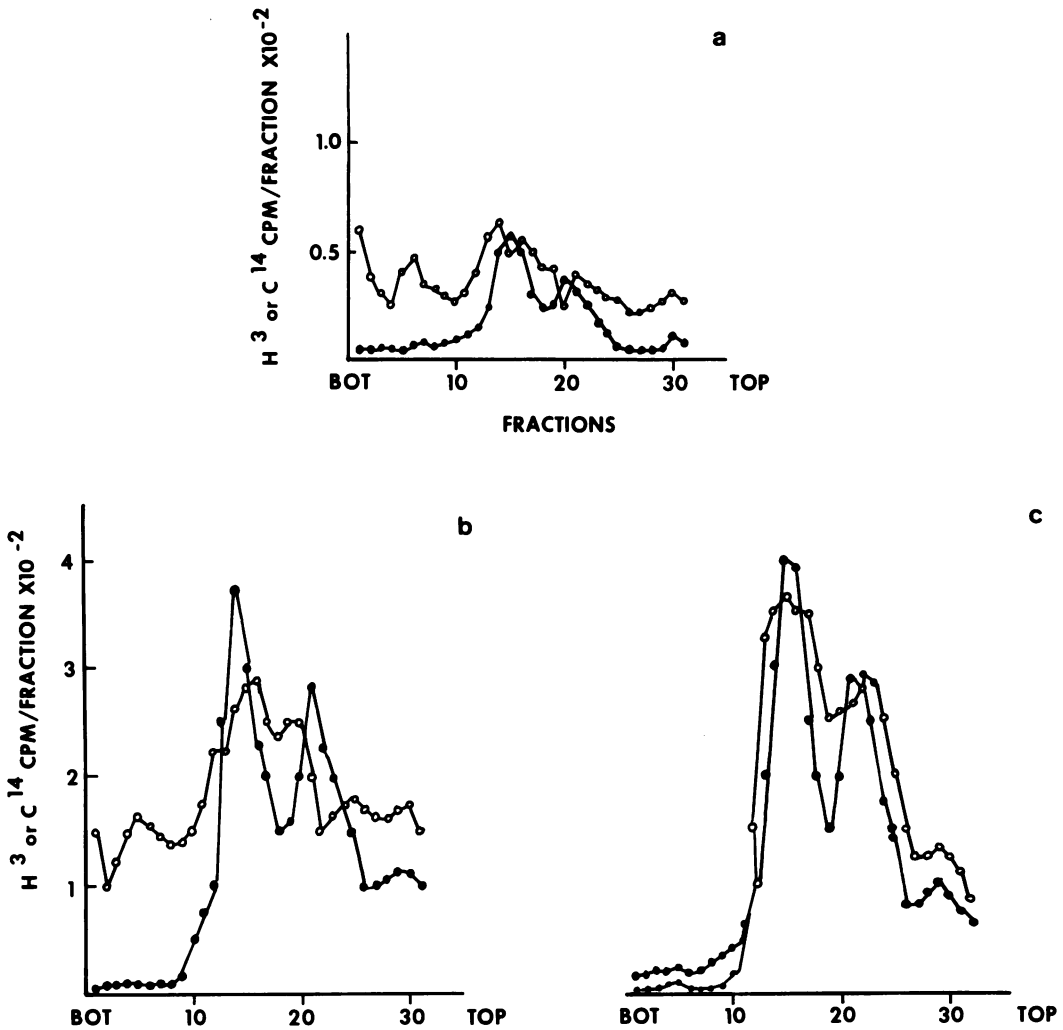


FIG. 6. *Analysis of RNAs contained in cytoplasmic structures from infected cells. Fractions representing various regions of the sucrose density gradient (Fig. 4b) were pooled and used for the isolation of the RNAs. Samples containing the RNAs were analyzed by centrifugation on 16-ml gradients of 5 to 20% sucrose in TES at 22,500 rpm for 14 h at 4 C in an SW27.3 rotor. a, RNAs from fractions 1 through 10, (b) RNAs from fractions 11 through 20 and (c) RNAs from fractions 21 through 28. Symbols:* ○, $^{14}C$; ●, $^{3}H$.

They sedimented in the gradient at rates of 28*s* and 18 to 15*s*. Also a minor species of RNA, with a sedimentation rate of 4*s*, is present.

The viral RNAs were analyzed by electrophoresis on polyacrylamide gels. The results of such an analysis are presented in Fig. 7. The heterogeneity in the types of viral RNAs found in the various virus-specific structures is similar to that observed in the previous section. Here again the viral RNAs obtained from the subcellular structures representing the ribosome-messenger RNA complexes (pool 3, Fig. 4b) migrated in polyacrylamide gels with mobilities almost similar to those of the host ribosomal RNAs (Fig. 7c). RNA with electrophoretic mobility similar to that of the 42*s* form of viral RNA was absent. The failure to observe the 42*s* form of viral RNA was not due to its absence in the cytoplasmic extract of the infected cells since the results presented in Fig. 7a and 7b indicate that 42*s* form of RNA is one of many species of viral RNAs present in the rapidly sedimenting virus-specific structures. Thus the RNA-ribosome complexes found in the infected cells contain two types of RNAs psossessing sedimentation rates and electrophoretic abilities resembling the host ribosomal RNAs. The results presented in the following section confirm

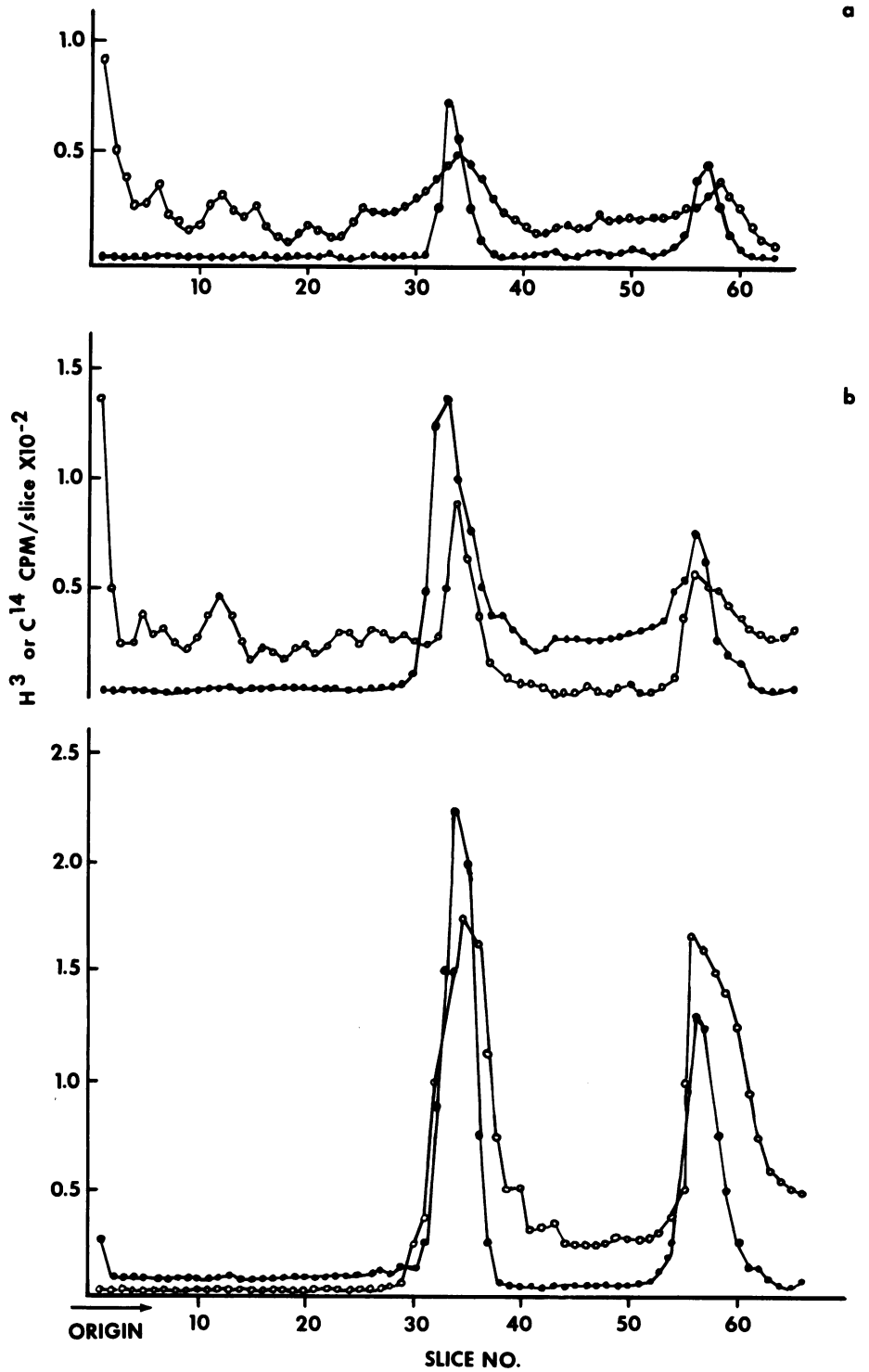


FIG. 7. *Analysis of RNAs by electrophoresis on polyacrylamide gels. Samples of RNAs identical to those used for analysis in Fig. 6a, b, and c were used for electrophoretic analysis. Electrophoresis was carried out on 2.2% polyacrylamide-agarose gels at 6 mA/gel for 2.5 h at 25 C. Electropherograms a, b, and c represent results obtained with samples of RNAs used for analysis on Fig. 6a, b, and c, respectively. Symbols:* ○, $^{14}C$; ●, $^{3}H$.

that the above two types of RNAs isolated from the ribosome complexes are viral and not cellular in origin.

The results presented in Table 1 show the base composition of the 28s and 18 to 15s viral RNAs obtained from messenger RNA-monosome complexes. Virus-specific structures were radiolabeled with $^{32}$P-orthophosphate under conditions similar to those described in the previous sections. Viral RNAs were isolated from the ribosome-RNA complexes present in the cytoplasmic extract and analyzed by centrifugation on sucrose density gradients. Fractions representing peaks of radioactivity were pooled and used for analysis. The results indicate that adenine is the most predominant base present in the 28s and 18 to 15s species of RNAs. The amount of adenine present in the viral messenger RNAs is distinctly higher than that present in the 42 or 26s form of viral RNAs. The results on the base composition analysis also indicate that the 28s and 18 to 15s forms of RNAs found in the ribosome-RNA complexes are not cellular RNAs since HeLa cell ribosomal RNAs possess a high guanine and cytosine content (10).

Additional evidence was sought to establish the nature of the RNAs associated with ribosomes in infected cells. The RNAs, labeled with $^{3}$H-uridine, were isolated from the ribosome-RNA complexes by methods similar to those described under Fig. 4b and 6c. The RNAs were tested for their capacity to anneal with unlabeled double-stranded RNA from infected CE cultures. Double-stranded viral RNA was isolated from chicken embryo cells infected with SB virus. The double-stranded RNA was denatured by heating at 100 C in 0.1 × SSC and then annealed with radioactive viral RNAs in the presence or absence of excess of unlabeled viral or cellular RNAs (Table 2).

Annealing the labeled 42s RNA with unlabeled double-stranded SB RNA rendered 8.9% of the input label resistant to ribonuclease. The conversion of the labeled 42s RNA into a ribonuclease resistant form was inhibited in the presence of unlabeled 42s RNA. Unlabeled HeLa cell ribosomal RNA had no such effect. This indicated that the annealing as observed here was specific for SB viral RNA. Both types of RNA (28s and 18 to 15s) isolated from the ribosome-RNA complexes annealed with the double-stranded form of viral RNA. Unlabeled 42s RNA was able to compete in the above annealing process, whereas HeLa cell ribosomal RNA had little effect. The above results indicate that the RNAs associated with the ribosomes are virus-specific and may not represent cellular RNAs.

## DISCUSSION

At present little experimental evidence exists on the type of RNA acting as messenger in arbovirus-infected cells. Picornaviruses contain infectious RNA, and it has been clearly demonstrated that this RNA is the type associated with polyribosomes in infected cells (44). Such direct correlation between the infectivity and the capacity of the viral RNA to act as messenger is lacking in the arbovirus system. Some of the problems involved in the isolation of virus-specific polyribosomes and messenger RNA from arbovirus-infected cells were circumvented in the present study by the use of HeLa cells and ts2, a temperature-sensitive mutant of SB virus. The rationales behind the use of inhibitors like cycloheximide and sodium fluoride are presented in the introduction. The conclusions drawn from

TABLE 1. *Base composition of viral RNAs*

| Type and source of RNA[a] | Percent | | | |
|---|---|---|---|---|
| | Adenine | Uracil | Guanine | Cytosine |
| 28s viral RNA from RNA-ribosome complex | 31.0 ± 1.5 | 19.0 ± 0.5 | 28.1 ± 1.0 | 21.1 ± 1.2 |
| 18 to 15s viral RNA from RNA-ribosome complex | 33.0 ± 1.5 | 20.8 ± 1.0 | 21.7 ± 2.0 | 23.8 ± 1.5 |
| 42s RNA from virion | 26.2 ± 2.0 | 22.1 ± 1.0 | 26.8 ± 1.5 | 26.1 ± 1.0 |
| 26s RNA from infected cells | 25.9 ± 1.6 | 21.4 ± 1.1 | 26.6 ± 1.0 | 26.1 ± 1.0 |

[a] Viral RNAs from the RNA-ribosome complexes contained in infected cells were prepared by methods identical to those described in legends for Fig. 4 and 6. $^{32}$P-orthophosphate was used for radiolabeling the viral RNAs. The viral RNAs were isolated and analyzed by methods identical to those shown in the legend for Fig. 6c. Appropriate fractions were pooled, and the base compositions of the RNAs were determined. $^{32}$P-labeled 42s and 26s forms of viral RNAs were prepared from mature virion and infected cells, respectively.

TABLE 2. *Annealing of RNAs from the ribosome-RNA complexes to double-stranded viral RNA*[a]

| Types of RNAs present during annealing in the samples | | | RNase-resistant radioactivity (%) remaining after | |
|---|---|---|---|---|
| Single-stranded RNA | | SB virus-specific double-stranded RNA (0.05 μg) | | |
| $^3$H-uridine-labeled viral RNA | Single-stranded RNA, unlabeled (1.0 μg) | | Denaturation | Annealing |
| 42*s* from SB virions | None | Absent | 0.20 | 0.24 |
| | None | Present | 0.32 | 8.90 |
| | 42*s* from SB virions | Present | 0.32 | 0.35 |
| | 28*s* and 18*s* HeLa ribosomal RNAs | Present | 0.30 | 8.90 |
| 28*s* from ribosome-viral viral RNA complex | None | Absent | 0.16 | 0.20 |
| | None | Present | 0.15 | 7.50 |
| | 42*s* from SB virions | Present | 0.15 | 0.10 |
| | 28*s* and 18*s* HeLa ribosomal RNAs | Present | 0.15 | 7.60 |
| 18 to 15*s* from ribosome-viral RNA complex | None | Absent | 0.20 | 0.25 |
| | None | Present | 0.15 | 6.90 |
| | 42*s* from SB virions | Present | 0.25 | 0.20 |
| | 28*s* and 18*s* HeLa ribosomal RNAs | Present | 0.15 | 6.70 |

[a] The double-stranded viral RNA was prepared from SB virus-infected CE cells, and 0.05 μg was used per sample. Unlabeled or labeled 42*s* RNA was obtained from purified SB virions. Labeled RNAs originating from ribosome-RNA complexes were obtained by methods similar to those described in the legend for Table 1. The input-labeled RNA per sample was 16,000 cpm for 42*s*, 12,500 cpm for 28*s*, and 8,500 cpm for 18 to 15*s* forms of viral RNAs. The ribonuclease-resistant radioactivity in samples was determined immediately after denaturation and after annealing by exposing samples to ribonuclease. The results presented are the average of three independent determinations. The variation in each value from experiment to experiment was less than 1.0%.

experiments involving the use of these inhibitors are based on the current knowledge of their mode of action. It may be argued that the results obtained with the mutant virus may not be representative of infection with the wild type. However, the mutant ts2 behaved similarly to the HR virus with respect to the production of infectious virus and the synthesis of viral RNAs (Fig. 1 and 2). Thus conclusions drawn from studies using the mutant are probably of general application. The 28*s* and 18 to 15*s* species of RNAs probably represent viral RNAs possessing messenger function since they were isolated from complexes containing ribosomal structures. Such a conclusion is based on analogous studies used by Vesco and Colombo (45) for the isolation and characterization of messenger RNAs from uninfected HeLa cells. The above authors observed the accumulation of RNA-monosome complexes in HeLa cells subsequent to the disaggregation of polyribosomes by sodium fluoride. The RNAs associated with the above complexes behaved like the polysome-associated RNA and contained the predominant classes of HeLa cell messenger molecules (45). Similar results were also reported by Lebleu et al. (27) who studied the distribution of 9*s* RNA (considered to be the hemoglobin messenger) with single ribosomes in reticulocytes incubated with sodium fluoride. Thus we used sodium fluoride to disaggregate polyribosomes and to accumulate RNA-ribosome complexes in the infected cells. Incubation of uninfected HeLa cells with cycloheximide and sodium fluoride decreased the level of polyribosomes to about 50% as compared to that in untreated cells (data not presented). The results obtained from experiments involving the analysis of cytoplasmic extracts by centrifugation on CsCl (Fig. 5) indicated that ribosome-RNA complexes isolated from the infected cells under the present conditions were indeed monosome-RNA complexes.

The major species of single-stranded viral RNAs found in SB virus-infected HeLa cells possess sedimentation rates of 42, 26, and 15*s*, respectively. However, the RNAs isolated from the ribosome-RNA complexes possessed sedimentation rates of 28*s* and 18 to 15*s*. Additionally, the mobilities of the above RNA species in polyacrylamide gels were more or less similar to

those of ribosomal RNAs. Apparently the above observations may suggest that RNAs isolated from the ribosome complexes may be cellular RNAs. However, such a contention is not valid since the results obtained from the hybridization experiments as well as those from the analysis of base composition indicate that the RNAs are indeed virus specific. Additionally it may be mentioned in this context that actinomycin D was used to inhibit the synthesis of cellular RNAs subsequent to infection. Thus it seems unlikely that the RNAs isolated from the ribosome complexes are cellular in origin. At present the apparent differences in the sedimentation rates and electrophoretic mobilities of the viral messenger RNAs (28*s* and 18 to 15*s*) and the 26 and 15*s* species is unclear. However, the amount of adenine present in the 28*s* messenger RNA species is 6 to 7% higher than that found in 26 or 42*s* RNAs. The recent observation of Johnston and Bose (25) on the presence of an adenylate-rich segment in the RNA of SB virus was similar to those findings observed with many mammalian messenger RNAs (11, 12, 14, 28). Preliminary experiments indicate that adenylate-rich segments can be isolated from the 28*s* and 18 to 15*s* species of viral RNAs. The poly A segments isolated from the viral RNAs possess a sedimentation rate of 2 to 2.5*s*. The addition of poly A segments to single-stranded RNAs may change their sedimentation rates and electrophoretic mobilities. However, the extent of such a change is unknown at present. Thus it is difficult to establish from the present data that observed viral messenger RNAs (28*s* and 18 to 15*s*) are indeed the 26 and 15*s* species containing poly A segments.

The RI form of Semliki Forest virus has been reported to be associated with polyribosomes in the infected cells (18). The above results are at variance with those reported here. It is conceivable that the sucrose density gradient fractions representing viral polysomes as used in the above report may have been contaminated with viral replicative complexes. The replicative complexes contain RI form of viral RNA (40). Possibly the RI form of viral RNA apparently associated with viral polysomes may originate from the contaminating replicative complexes. Little or no ribonuclease-resistant RNA was associated with the messenger RNA-ribosome complexes described here, indicating that RI may not act as messenger in SB virus-infected cells. Such a contention is further supported by the observation on the kinetics of viral proteins in vivo. Viral polypeptides appearing in arbovirus-infected cells at different times after infection are identical (18, 35, 43). Englehardt et al. (15) reported that besides MS2 phage RNA, the RI forms of viral RNA also can act as messenger. However, the relative amounts of the different proteins synthesized varied depending upon the type of RNA used as the messenger. Based on the above observations, one would expect preferential synthesis of only certain classes of viral polypeptides in vivo if the RI forms of viral RNA act as messengers. Clearly this is not true in SB or SF virus-infected cells. Thus it appears unlikely that RI forms of SB viral RNA function as messengers in vivo.

The present results do not rule out the possibility that the other forms of viral RNAs can act as messengers in infected cells. It could be argued that the two species of RNAs reported here as messengers represent breakdown products of the virion RNA (42*s*) generated during isolation procedure. Such a contention appears improbable since there was little heterogeneity in the ribosomal RNAs isolated from the ribosomes contained in the virus-specific structures. The observation that a 28*s* and 18 to 15*s* species of viral RNAs serve as messengers in SB virus-infected cells is surprising since the virion RNA (42*s*) is infectious. The latter implies that the virion RNA should serve as a messenger in some form at least early during infection. It is not known whether the whole viral genome or only part of it is translated during the early part of infection. In this context, it is pertinent to cite Friedman's (17) observation that the RNA from the infecting virus was converted from a 42*s* to a 26*s* form within 30 min after infection. If the above observation is true, it is possible that the conversion of 42*s* RNA into the 26*s* species may be necessary for messenger activity. However, no direct evidence exists to support the above thesis. Further experimental work dealing with the fate of the parental RNA in the infected cells is necessary to characterize the form of viral RNA serving as the initial messenger in infected cells.

## ADDENDUM

While the present work was being completed, Kennedy (Biochem. Biophys. Res. Commun. **48**:1254–1258, 1972) reported that the majority of the viral RNA associated with polysomes in Semliki Forest virus-infected chicken embryo cells consisted of a 26*s* form. Trace amounts of a 33*s* form of viral RNA were also detected. The above results are in partial agreement with those reported here. No 15 to 18*s* form of viral RNA was found on the polysomes, a finding different from that reported in the present work. The above difference may be due to the conditions used by

Kennedy to analyze the viral RNA by electrophoresis, which may not permit the detection of a 15 to 18*s* species of viral RNA.

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