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Messenger Ribonucleoprotein Complexes Containing *in vitro*-Synthesized 26S and 42S Semliki Forest Virus RNA

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With 9 Figures

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Summary

An extract derived from Semliki Forest virus (SFV) infected cells is described which catalyzes the synthesis of virus-specific RNAs. The newly-synthesized 26S and 42S RNAs are found complexed with protein as messenger ribonucleoproteins (mRNPs). These mRNPs either are non-membrane bound or are associated with large cytoplasmic lipoprotein membranes, and they are found as free mRNPs as well as mRNPs bound to ribosomal subunits, ribosomes, and polysomes. Following treatment with Tween 40 and deoxycholate, membrane-bound mRNPs containing *in vitro*-synthesized 26S RNA are dissociated and sediment at 33S. These membrane-dissociated mRNPs contain relatively little protein. In contrast, the free or ribosome-bound mRNPs, which are isolated as 30S to 160S particles, remain heterogeneous after detergent treatment and have a much higher protein content. Addition of purified, native 40S ribosomal subunits to the extract leads to the formation of complexes between the added ribosomal subunits and the newly-synthesized viral mRNA. The *in vitro*-synthesized 26S and 42S RNAs participate in the assembly of translational initiation and elongation complexes.

Introduction

Regardless of the cell substrate used, two major virus-specific single-stranded RNAs, which sediment at 42S and 26S, are regularly synthesized in Semliki Forest and Sindbis virus-infected cells. The infectious 42S species is identical to the genomic viral RNA (mol. wt. 4×10^6; refs. 26, 38), and the 26S RNA (mol. wt. 1.6×10^6; refs. 24, 38) is derived from the 3′ end of the genomic RNA (12, 36, 44). Both species can be found on polysomes, and the 26S RNA has been shown to be the major mRNA late in infection (14, 20, 30, 35, 39).

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In extracts from SFV- or Sindbis virus-infected cells, two classes of virus-specific ribonucleoproteins (RNPs) have been identified (7, 16, 35). One is the viral core consisting of the 42S RNA and the core protein; it sediments between 140S and 150S (1, 7). The second class is obtained from polysomes by disruption with EDTA which releases the mRNA as a mRNP sedimenting with about 65S (28, 30).

Using an *in vitro* system which synthesizes both species of SFV RNAs, we have shown earlier that the 42S as well as the 26S RNAs accumulate for at least 180 minutes (28). The *in vitro*-synthesized single-stranded 42S and 26S RNAs are released during synthesis from the membrane-associated enzyme-template complex and are found in structures having sedimentation coefficients higher than those expected for deproteinized 42S and 26S RNA, respectively. We have shown earlier that our *in vitro* system contained besides nuclei, mitochondria, and microsomes also large amounts of free and membrane-bound polysomes (28). The possibility existed therefore that the crude enzyme-template complex used represented a coupled system catalyzing not only the synthesis of all viral RNAs but also allowing the formation of translational initiation and elongation complexes.

In the present study we have isolated and partially characterized mRNPs containing *in vitro*-synthesized 42S and 26S RNAs. The results indicate that at least some of these newly made RNAs are participating in the translational machinery provided by the cellular extract.

Materials and Methods

Cells and Virus

The procedure for culturing the BHK-21 cells, for virus assay, and for growing stock SFV in BHK-21 cells has been described (19).

Preparation of Cellular Extracts

The growth medium and the procedure for infecting BHK cells with SFV also have been described (28). The cells from 6 to 10 cylindrical glass bottles (660 cm^2/bottle), each containing approximately 10^8 cells, were used as the source of enzyme. At 4 hours after infection, the maintenance medium containing 0.15 μg actinomycin-D per ml was removed and the cells were washed first with ice-cold PBS-PVS [phosphate-buffered saline (13) containing polyvinyl sulfate, 20 μg/ml], and subsequently with RSB-PM (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$ containing 1 mM 2-mercaptoethanol, and polyvinyl sulfate 20 μg/ml). The cells were scraped into RSB-PM and disrupted with 25 strokes of a tight-fitting Dounce homogenizer. To obtain a maximum of material and to prevent damage of the viral replication complex, the crude enzyme-template complex was prepared in two steps: The homogenate was centrifuged for 10 minutes at $250 \times g$ to separate it into two fractions: i) a cytoplasmic extract; and ii) a large-particle fraction that sedimented as a pellet. It has been shown earlier that the large-particle fraction contained nuclei, mitochondria, and rough and smooth endoplasmic reticulum, whereas the cytoplasmic extract contained all of the above components except for nuclei and mitochondria (28). The cytoplasmic extract, totaling approximately 40 ml, was subjected to high-speed centrifugation (SW 41 rotor, 30 minutes at $185{,}000 \times g$). The resulting pellet was resuspended and combined with the large-particle fraction in 10 ml RSB-PM to give the crude enzyme-template complex.

Isotope Labeling

To label cellular RNA prior to infection with SFV, 5-^{3}H-uridine (4 μCi/ml, 26 to 28 Ci/mmol) was added for 6 hours to BHK cells in their logarithmic phase of growth

(approximately 50 per cent confluent monolayers). The radioactive label was removed, the cells were washed three times with prewarmed growth medium, and incubation was continued until the cultures reached confluency and were ready for infection (12 to 16 hours after labeling).

Assay Conditions for Synthesizing Viral RNAs and mRNP Complexes

The standard reaction mixture for the *in vitro* synthesis of single- and double-stranded viral RNAs under optimal conditions has been described (28). For the formation of mRNP complexes it was modified by the addition of unlabeled methionine (10 μmol/ml). GTP and UTP (both labeled with ^{32}P in the α-position) had specific activities of 42,000 and 25,000 counts per minutes per nmol, respectively.

Preparation of mRNPs by Sucrose Density Gradient Centrifugation

At the end of the *in vitro* reaction, the mRNP complexes not bound to membrane were separated from the crude enzyme-template complex by low-speed centrifugation for 10 minutes at $250 \times g$ and were collected in supernatant 1. Membrane-bound 26S SFV RNA was released by treating the resulting pellet (pellet 1) with Tween 40-DOC detergent mixture (0.8 per cent Tween 40 and 0.4 per cent deoxycholate in RSB-PM) and was collected in supernatant 2 after centrifugation for 10 minutes at $250 \times g$. The released material in supernatant 1 and 2, respectively, was layered onto replicate 15 to 30 per cent (w/w) sucrose gradients (36 ml) in RSB and was centrifuged in a SW 27 rotor at $38,000 \times g$ for 14 to 17 hours at 4° C. Fractions of 1 ml each were collected from the top of the gradient. To 100 μl portions was added 50 μg of bovine plasma albumin as carrier, and the samples were made 5 per cent in trichloroacetic acid. The resulting acid-insoluble material was collected on membrane filters, and the amount of radioactivity on the dried filters was determined as described earlier (28).

Isolation of Polysomes From Supernatant 1

Supernatant 1 was treated with Tween 40-DOC (0.8 and 0.4 per cent, respectively) and then was layered onto preformed discontinuous sucrose gradients made of 3 ml of 2.1 M sucrose in RSB and 2 ml of 1.6 M sucrose in RSB. Centrifugation was at 4° C for 18 hours at $185,000 \times g$ in a SW 41 rotor and the polysomes were collected in the pellet (5).

Preparation of mRNPs Derived From Polysomes

Polysomes were dissociated by addition of either 30 mM EDTA (18, 21, 23) or 1 mM puromycin-500 mM KCl (4, 6). For the latter method, the pelleted polysomes were resuspended in a buffer containing 500 mM KCl, 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.6, 1 mM puromycin, and 1 mM dithiotreitol and kept for 10 minutes at 0° C, and then 10 minutes at 37° C. The puromycin-treated polysomes were layered onto 5 to 20 per cent sucrose gradients containing 500 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.6. Centrifugation was in a SW 41 rotor at $185,000 \times g$ for three hours at 22° C.

Isolation of Native ^3H-Labeled 40S Ribosomal Subunits ($40S_n$)

BHK cells in their logarithmic phase of growth were labeled with 2 μCi ^3H-uridine per ml as indicated in the section "Isotope labeling". $40S_n$ particles were prepared according to PAIN and HENSHAW (31).

CsCl Density Gradient Analysis of Fixed mRNP Particles

A modification of the technique described by BALTIMORE and HUANG (2) was used. Samples containing mRNPs were adjusted to 6 per cent final glutaraldehyde that had been neutralized with 1 M $NaHCO_3$ immediately before use and were left at 0° C for at least 1 hour. The samples were layered onto 5 ml preformed CsCl density gradients made in buffer containing 25 mM KCl, 20 mM triethanolamine-HCl, pH 7.2, 2 mM magnesium acetate, 1 mM dithiotreitol, 1 mM EDTA, and 0.8 Brij 35. The gradients were centrifuged in a SW-41 rotor at $160,000 \times g$ for 16 to 20 hours at 4° C. Fractions, each containing 0.16 ml, were collected from the top of the gradient. Samples of 5 μl each were taken to determine the refractive index, and the radioactivity of each fraction was determined as described above.

Cs_2SO_4-Dimethyl Sulfoxide Gradient Centrifugation

The density distribution of mRNPs with a low protein/RNA ratio was determined in Cs_2SO_4-dimethyl sulfoxide gradients according to LINDBERG and SUNDQUIST (25).

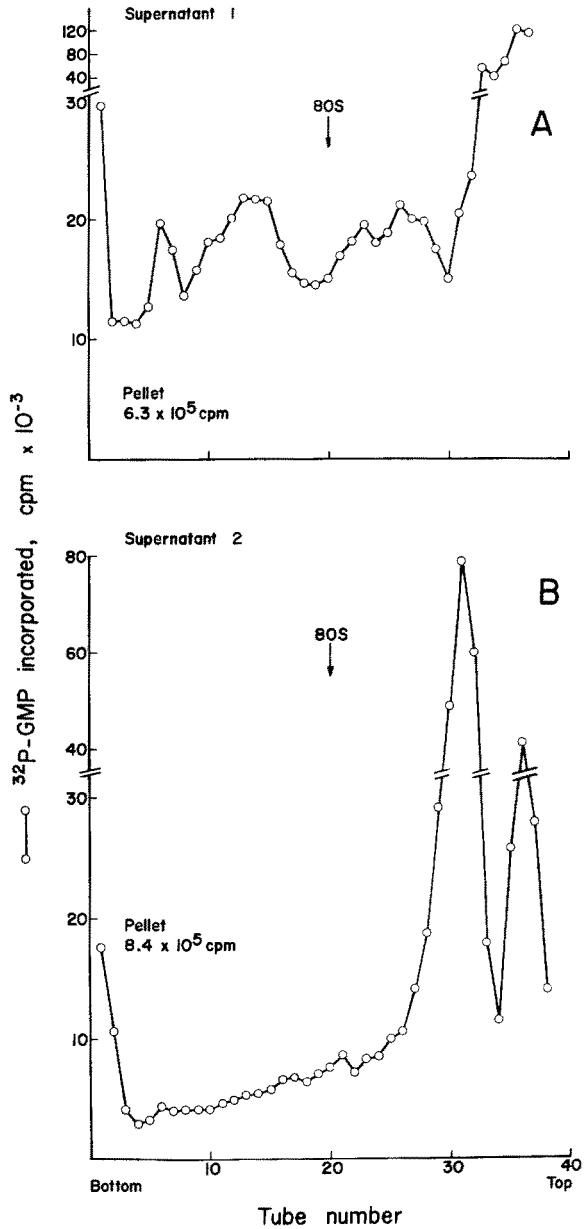

Fig. 1. Association with cytoplasmic structures of SFV RNAs synthesized *in vitro* by the crude enzyme-template complex obtained from infected BHK cells. BHK-21 cells, harvested 4 hours after infection, were disrupted by Dounce homogenization and centrifuged for 10 minutes at $250 \times g$ to yield a pelleted, large particle fraction and a

Results

Association of the in vitro-*Synthesized SFV RNAs With Cytoplasmic Structures*

The first step toward characterizing the complexes containing *in vitro*-synthesized SFV RNAs was to determine their distribution between the soluble cytoplasm and cytoplasmic membranes of the cell extract. The crude enzyme-template complex (see "Materials and Methods" section) prepared at 4 hours after infection was incubated for 30 minutes in the presence of ^{32}P-GTP. At the end of the reaction, the structures containing the *in vitro*-synthesized SFV RNAs were sequentially removed from the crude enzyme-template complex in two steps: i) large structures were pelleted by differential centrifugation at low speed to yield supernatant 1, in which all structures not bound to membranes were collected; and ii) the structures associated with cytoplasmic lipoprotein-membranes of large size and present in the pellet were solubilized in Tween 40-deoxycholate (DOC) and upon recentrifugation were collected in supernatant 2. Panel A of Figure 1 shows the sedimentation profile of the non-membrane bound structures that were separated from the crude enzyme-template complex by low-speed centrifugation (supernatant 1). The *in vitro*-synthesized viral RNA was found in structures of five main classes by size: i) sedimenting slower than 30S; ii) 30S to 80S; iii) 100S to 130S; iv) 140S to 160S; and v) structures sedimenting faster than 175S. The latter structures, which are collected in the pellet under the experimental conditions used, were identified in the electron microscope as smooth and rough endoplasmic reticulum of small size (M. R. MICHEL, unpublished observation). That the differently sized structures containing the newly-synthesized RNA were not lipoprotein membranes could be shown by Tween 40-DOC treatment: The detergent treatment of supernatant 1 prior to its centrifugation on sucrose gradients did not change its sedimentation profile (data not shown). Panel B of Figure 1 shows the sedimentation profile of the structures containing *in vitro*-synthesized viral RNA that were released from their membranous association with the Tween 40-DOC (supernatant 2). The detergent treatment released a complex with an average sedimentation coefficient of 33S.

Identification of Viral RNAs

Selected fractions of the differently sized cytoplasmic structures shown in Figure 1A and 1B were extracted with the SDS-Brij-EDTA detergent mixture and the RNAs were analyzed on replicate sucrose gradients (Figs. 2 and 3).

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cytoplasmic extract. The material present in the cytoplasmic extract was pelleted at 185,000 $\times g$ for 30 minutes, resuspended together with the large particle fraction in RSB-PM and the resulting crude enzyme-template complex was incubated at 28° C for 30 minutes in the assay mixture containing 10 μmol methionine per ml and ^{32}P-GTP. After that time, cycloheximide (100 μg/ml) was added for 1 minute to prevent a possible run-off of ribosomes. The sample was then rapidly chilled to 0° C and centrifuged at 250 $\times g$ for 10 minutes to yield supernatant 1. The remaining material was resuspended in RSB-PM containing Tween 40 and DOC and recentrifuged at 250 $\times g$ for 10 minutes to yield supernatant 2. Supernatant 1 and 2 were layered over separate 15 to 30 per cent sucrose gradients in RSB. Centrifugation was at 4° C for 14 hours in a SW 27 rotor at 38,000 $\times g$. Fractions were collected, absorbancy at 260 nm and radioactivity were determined as described in the "Materials and Methods" section. The 80S BHK ribosomes served as sedimentation marker

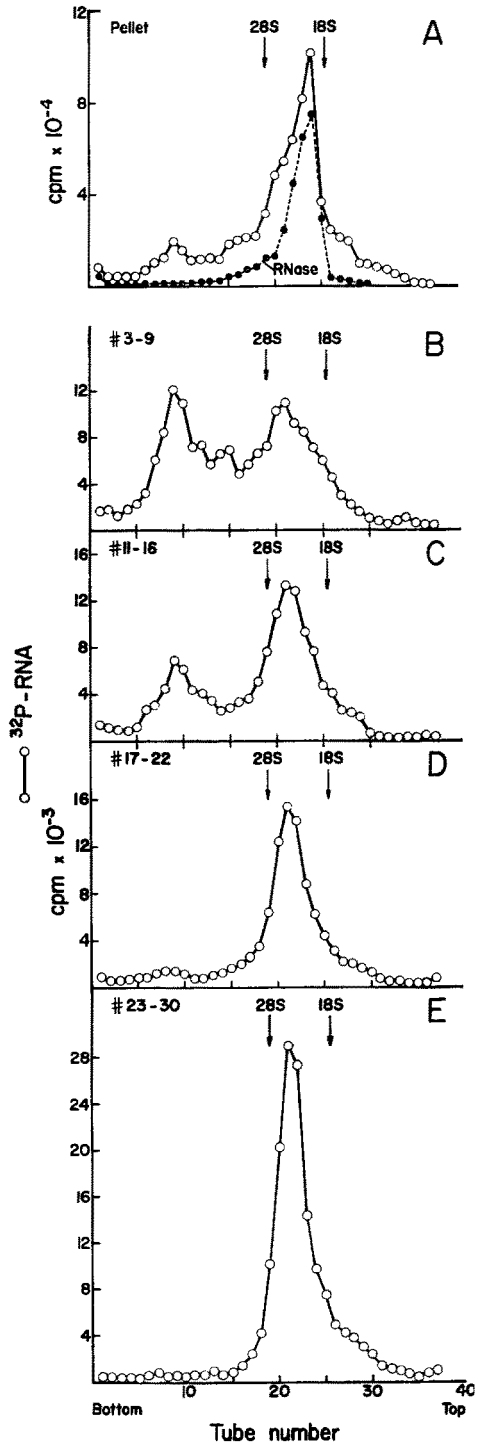
Pellet
28S
18S
A
cpm x 10⁻⁴
RNase
#3-9
B
#11-16
C
#17-22
D
#23-30
E
32P-RNA
cpm x 10⁻³
Bottom
Top
Tube number

Fig. 2

Figure 2 shows the sedimentation analysis of the RNAs extracted from the differently sized structures of supernatant 1. Three classes of newly-synthesized SFV RNAs were found: i) double-stranded (ds) RNAs and replicative intermediates (RIs) (28); ii) 26S single-stranded (ss) RNA; and iii) 42S ss RNA. The pellet as shown by RNase resistance, contained *in vitro*-synthesized ds RNA and RIs. They were associated originally with the small membranes that remained in supernatant 1 after low speed centrifugation (28). A substantial amount of 42S RNA and a small amount of 26S RNA were also present in this fraction. The *in vitro*-synthesized 26S RNA associated with non-membrane bound structures was distributed over the entire original gradient (tubes 3 through 30 of Fig. 1A), but predominantly in the 30S to 80S region (tubes 23 through 30). In contrast, the 42S RNA was found predominantly in structures sedimenting from 100S to 160S (tubes 3 through 16). The labeled material which originally sedimented at 30S and lower (tubes 31 through 34) contained some 26S RNA and RNA with sedimentation coefficients of 4S or lower (data not shown). As shown in Figure 3, supernatant 2 contained only two types of SFV RNAs: i) ds RNA and RIs that remained associated with cytoplasmic membranes after the Tween 40-DOC treatment; and ii) 26S RNA which was released by the detergent treatment from its association with large cytoplasmic lipoprotein membranes as a 33S complex (Fig. 3B, tubes 29 through 33). The shoulder of radioactivity trailing between the 33S complex and 175S in the original gradient of Figure 1B was due to structures also containing 26S RNA in small amounts (data not shown). It should be emphasized that the RNAs shown in Figure 2B through 2E and in Figure 3B were RNase sensitive. All ds RNAs and RIs present in supernatants 1 and 2 were associated with membranes and sedimented faster than 250S. The results show that i) the ds RNA and RIs are associated with large as well as with small sized membranes which are not solubilized by Tween 40-DOC. ii) Both, 42S and 26S RNAs are found associated with non-membrane bound structures sedimenting between 30S and 160S. In addition, both 42S and 26S RNAs are also present on small-sized membranes which are resistant to detergent treatment. iii) A large amount of 26S RNA is also found on lipoprotein membranes of large

Fig. 2. Analysis by sucrose gradient centrifugation of the ^{32}P-RNAs synthesized *in vitro* and associated with the differently sized structures shown on panel A of Figure 1 (supernatant 1). The material in tubes 3 through 9, 11 through 16, 17 through 22, and 23 through 30 of panel A of Figure 1 were separately pooled. All fluids were made 20 mM in EDTA, 1.95 per cent in sodium dodecyl sulfate, and 0.5 per cent in Brij 35 (SDS-Brij-EDTA detergent mixture) and the RNAs were precipitated with 2 volumes of ethanol, and resuspended in TNE (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1 mM EDTA) before centrifugation. The RNA in the pellet of panel A of Figure 1 was extracted directly with the SDS-Brij-EDTA detergent mixture. All samples were layered over replicate 15 to 30 per cent sucrose gradients in TNE. Centrifugation was at 15° C for 16 hours in a SW 27 rotor at 76,000 × *g*. Fractions were collected, and each fraction was analyzed for absorbancy at 260 nm and radioactivity (solid line). For reasons of clarity, RNase-resistant RNA (broken line) is shown only for the fractions obtained from the analysis of pellet A. RNase treatment was at 3 μg/ml for 30 minutes at 37° C in RSB containing 250 mM potassium chloride and 25 mM potassium citrate. The untreated and RNase treated samples were processed for radioactivity as described in the "Materials and Methods" section. The 28S and 18S ribosomal RNAs served as sedimentation markers. Note the different scales for the radioactivity

size. These membranes are solubilized with Tween 40-DOC and release the 26S RNA in the form of a 33S particle.

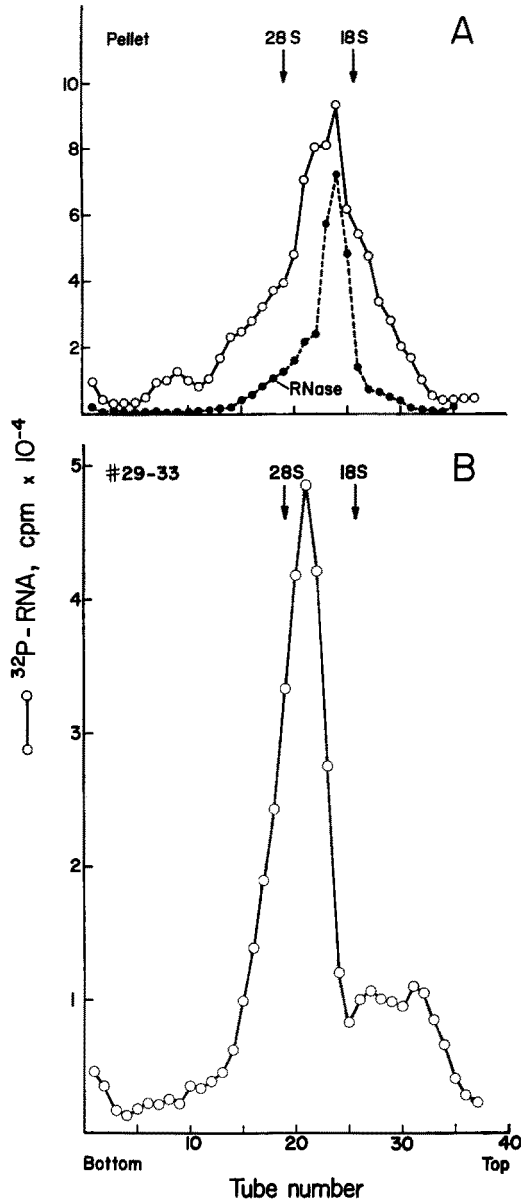

Fig. 3. Analysis by sucrose gradient centrifugation of the ^{32}P-RNAs synthesized *in vitro* and associated with the differently sized structures shown on panel B of Figure 1 (supernatant 2). Tubes 29 through 33 of panel B of Figure 1 were pooled and the RNAs of the pooled fractions as well as of the pellet of panel B of Figure 1 were extracted separately and analyzed as described in Figure 2. For reasons of clarity, RNase-resistant RNA (broken line) is shown only for the fractions obtained from the analysis of pellet B

Buoyant Density of mRNPs

RNA-protein complexes containing newly-synthesized RNA were pooled from gradients similar to that shown in Figure 1 A, fixed in glutaraldehyde, and banded on preformed CsCl density gradients. The RNA-protein complexes derived from the 90S to 120S region of supernatant 1 exhibited a broad density distribution ranging from 1.35 to 1.56 gcm^{-3} with a prominent peak at 1.49 gcm^{-3} (data not shown). The 70S to 90S and the 40S to 65S regions showed similar heterogeneous density profiles, although the relative amount of structures with densities smaller than 1.49 gcm^{-3} increased. Although they do not exhibit a simple density distribution, the *in vitro*-formed RNA-protein complexes may correspond to free mRNPs ($\rho = 1.35$ to 1.40 gcm^{-3}) and mRNPs associated with 40S ribosomal subunits ($\rho = 1.43$ gcm^{-3}) and ribosomes ($\rho = 1.47$ gcm^{-3}) (17, 21, 32, 33, 37).

When fixed in glutaraldehyde and banded in CsCl, 33S particles pooled from a sucrose gradient similar to that shown in Figure 1 B were found at the bottom of the gradient and in the pellet. When subjected to isopycnic banding in Cs_2SO_4-dimethylsulfoxide (DMSO) gradients this complex banded at 1.50 gcm^{-3} and was devoid of any ribosomal material. The 33S particle exhibited the same buoyant density even when not fixed in glutaraldehyde prior to Cs_2SO_4-DMSO gradient centrifugation.

Entry of in vitro-*Synthesized RNA in Translational Initiation and Elongation Complexes*

It appeared likely that some of the newly-synthesized RNA was associating with ribosomal subunits in the crude lysate to form translational initiation and elongation complexes. To demonstrate that the crude extract derived from SFV infected cells provided the translational machinery, ^{3}H-labeled native 40S ribosomal subunits ($40S_n$) were added to the assay mixture in the presence of ^{35}S-methionine. When the crude enzyme-template complex was immediately separated by low-speed centrifugation (10 minutes at $250 \times g$) and the supernatant analyzed by sucrose gradient sedimentation, the majority of the small ribosomal subunits was found to sediment at approximately 45S (data not shown). Some ^{3}H-label cosedimented with monoribosomes and disomes and some pelleted. After 3 minutes of incubation (Fig. 4A), one-third of the total tritium label confined to structures with sedimentation coefficients of 40S and higher was still present in the small ribosomal subunit, whereas two-thirds of the remaining tritium counts were distributed about equally among complexes sedimenting between 60S and 120S and structures found in the pellet. The 15-minutes reaction markedly reduced the free tritium-labeled 40S particles present in the reaction (Fig. 4B). During that time a concomitant increase of tritium label was found in structures sedimenting between 60S and 120S. A significant increase in the ^{35}S-label was found in complexes with sedimentation coefficients higher than 40S. Significant amounts of ^{35}S-met-$tRNA_f$ were recovered only from structures with sedimentation coefficients ranging between 40S to 60S [precipitable by cold, and made soluble in hot, trichloro acetic acid (31)]. Using BD-cellulose chromatography (40), unbound ^{35}S-labeled methionyl-$tRNA_f$ could be eluted with linear sodium chloride gradients from fractions 33 to 39 of both sucrose gradients A

and B of Figure 4. That the ^{35}S-met associated with structures sedimenting faster than 60S was incorporated into stable peptide linkages could be shown by the addition of puromycin to the reaction mixture. If puromycin (1 mg/ml) was added

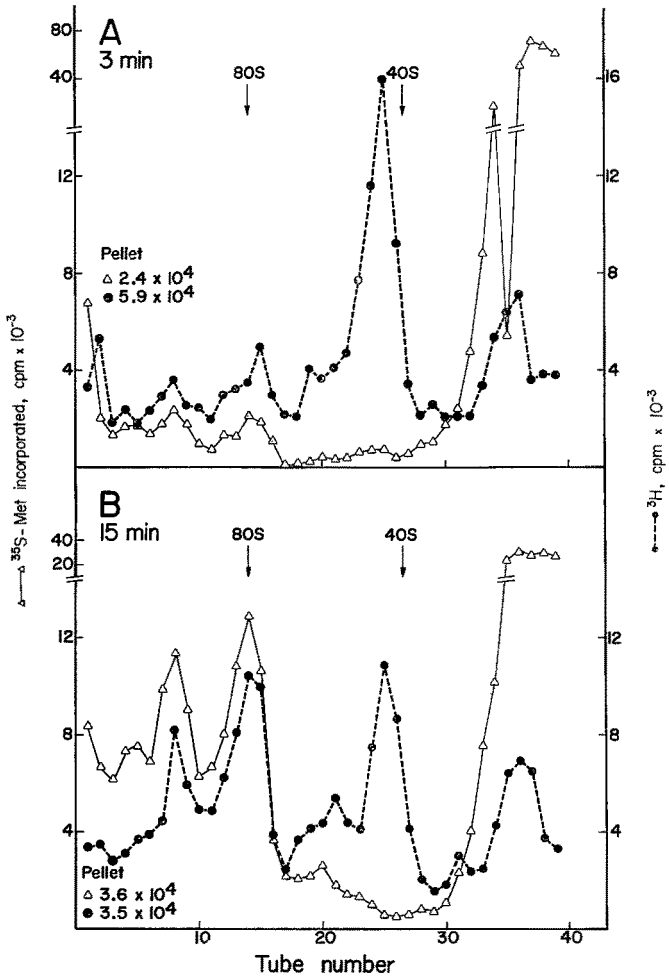

Fig. 4. Formation of ribosomal initiation and elongation complexes between $40S_n$ particles and ^{35}S-met. $40S_n$ particles labeled with tritium in their RNA moiety and the crude enzyme-template complex obtained from SFV infected cells were prepared as described in the "Materials and Methods" section. Each 2.4 ml of reaction mixture contained 7.2 A_{260} units of $40S_n$ particles and 0.27 mCi of ^{35}S-met (specific activity 280 Ci/mmol). After incubation, the samples were cooled to 0° C, supernatant 1 of the 3 minutes and the 15 minutes reaction was obtained by differential centrifugation at 4° C for 10 minutes at $250 \times g$, and was layered over separate 20 to 40 per cent sucrose gradients in a buffer containing 25 mM KCl, 20 mM triethanolamine-HCl, pH 7.2, 2 mM magnesium acetate, 1 mM dithiotreitol, and 1 mM EDTA. Centrifugation was at 4° C for 17 hours in a SW 27 rotor at $76{,}000 \times g$. Fractions were collected, and absorbancy at 260 nm and acid precipitable radioactivity were determined as described in the "Materials and Methods" section. 80S ribosomes and $40S_n$ particles spun in replicate gradients served as sedimentation markers. A: Incubation for 3 minutes. B: Incubation for 15 minutes

at time zero together with ^{35}S-met and 40S$_n$ particles to the reaction mixture, no ^{35}S-label was found in the 80S to 120S region of the gradient (data not shown). The initiator dipeptide ^{35}S-methionyl-puromycin which is released from the 80S complex could be determined by the extraction from the *in vitro* reaction with ethyl acetate (15). To detect any random aggregation of 40S$_n$ particles with soluble cellular components present in the assay mixture, confluent BHK-21 cells were mockinfected and incubated for 4 hours in the presence of 0.15 μg actinomycin-D per ml. The crude cellular extract was prepared as described in the "Materials and Methods" section. ^{3}H-labeled 40S$_n$ particles were added to the assay mixture in the presence of ^{35}S-met for the same time intervalls as shown in Figure 4. The sucrose gradient pattern showed a marked decrease in polysome function and

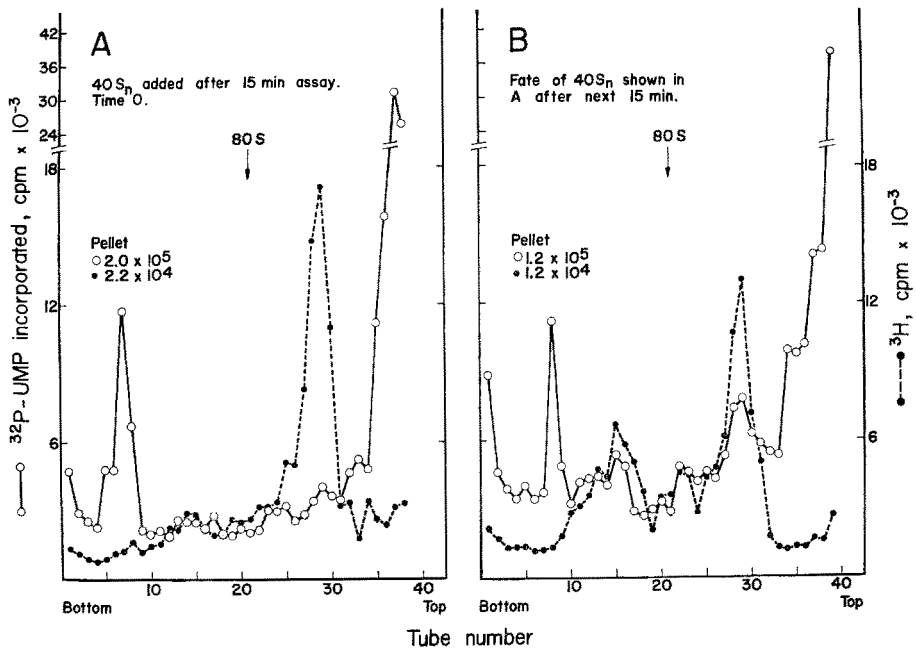

Fig. 5. Formation of ribosomal initiation and elongation complexes between 40S$_n$ particles and *in vitro*-synthesized 42S and 26S SFV RNAs. 40S$_n$ particles labeled with tritium in their RNA moiety and the crude enzyme-template complex were prepared as described in the "Materials and Methods" section. Each 2.4 ml of reaction mixture contained 6.8 A_{260} units of 40S$_n$ particles and 2.5 μmol of UTP labeled with ^{32}P in the α-position (specific activity 25,600 counts per minutes per nmol). The *in vitro* reaction preceded the addition of the 40S$_n$ particles by 15 minutes. At the times indicated the samples were cooled to 0° C, supernatant 1 of the 15 and 30 minutes reaction was obtained by differential centrifugation at 4° C for 10 minutes at 250 × *g*, and was layered over separate 15 to 30 per cent sucrose gradients in a buffer containing 25 mM KCl, 20 mM triethanolamine-HCl, pH 7.2, 2 mM magnesium acetate, 1 mM dithiotreitol, and 1 mM EDTA. Centrifugation was at 4° C for 14 hours in a SW 27 rotor at 38,000 × *g*. Fractions were collected, and absorbancy at 260 nm and acid precipitable radioactivity were determined as described in the "Materials and Methods" section. A: 40S$_n$ particles added after 15 minutes of *in vitro* reaction and immediately analyzed (time 0). B: Fate of 40S$_n$ particles added at zero time after next 15 minutes of incubation

a high level of free monoribosomes. No significant participation of the added $40S_n$ particles was observed in the assembly of initiation and elongation complexes. These results strongly suggested that translational initiation and elongation complexes can be formed in the crude extract derived from SFV infected cells.

To determine whether SFV RNA can enter these complexes, a similar experiment was performed by adding ^{3}H-labeled $40S_n$ particles to an *in vitro* reaction 15 minutes after the beginning of incubation in the presence of ^{32}P-UTP. The sedimentation profile of the labeled ribosomal subunits and the newly-synthesized RNA was determined immediately and after a further 15-minutes incubation (Figs. 5A and B).

In this experiment about half of the ^{3}H-labeled $40S_n$ particles entered into complexes sedimenting between 60S and 130S after 15 minutes incubation, and a significant fraction of the ^{32}P-labeled SFV RNA cosedimented in the same region.

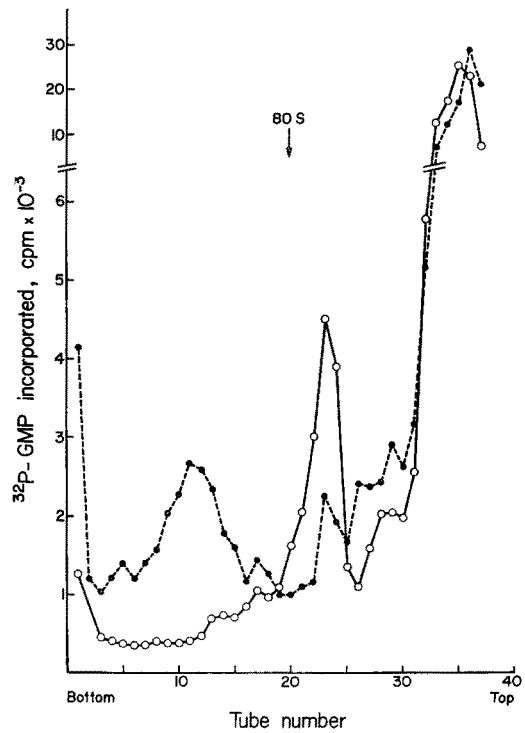

Fig. 6. Sensitivity toward EDTA of the non-membrane bound structures sedimenting in the polysomal region. The crude enzyme-template complex, prepared as described in the "Materials and Methods" section, was incubated for 30 minutes in the presence of ^{32}P-GTP. Supernatant 1 was obtained by differential centrifugation at 4° C for 10 minutes at $250 \times g$. Half of supernatant 1 was layered onto a 15 to 30 per cent sucrose gradient in RSB. The remaining half was treated with 30 mM EDTA and then layered onto a 15 to 30 per cent sucrose gradient in TNE. Centrifugation was at 4° C for 14 hours in a SW 27 rotor at $38{,}000 \times g$. Fractions were collected, absorbancy at 260 nm, and acid precipitable radioactivity were determined as described in the "Materials and Methods" section. The 80S BHK ribosomes served as sedimentation marker. Control (•); supernatant 1 treated with EDTA (○)

This suggests that at least some of the newly-synthesized viral RNA becomes associated with ribosomes in initiation and/or elongation complexes.

Sensitivity of RNA-Protein Complexes to EDTA and Puromycin

If the rapidly-sedimenting RNA-protein complexes are in fact viral RNA associated with one or more ribosomes, they should be dissociated by treatment with EDTA (18, 21, 23, 33). The experiment shown in Figure 6 shows that this is in fact the case. EDTA treatment of ^{32}P-labeled complexes dissociates them into a mRNP which sediments at 65S. Figure 7 shows that the intact 100S to 160S complexes have buoyant densities ranging from 1.45 to 1.56 gcm^{-3} in CsCl, densities expected for translational initiation and elongation complexes (37). The EDTA-treated complexes, on the other hand, band at 1.40 gcm^{-3}, typical for ribosome-dissociated mRNPs (37).

That the structures sedimenting faster than 80S were polysomes was shown by the addition of puromycin to the reaction mixture. The crude enzyme-template complex was incubated for 25 minutes in the presence of ^{35}S-met and then exposed to puromycin for 5 minutes to release the nascent peptide chains from polysomes. Figure 8 shows that the bulk of the radioactivity associated with large structures was released by puromycin, indicating that the large structures were polysomes.

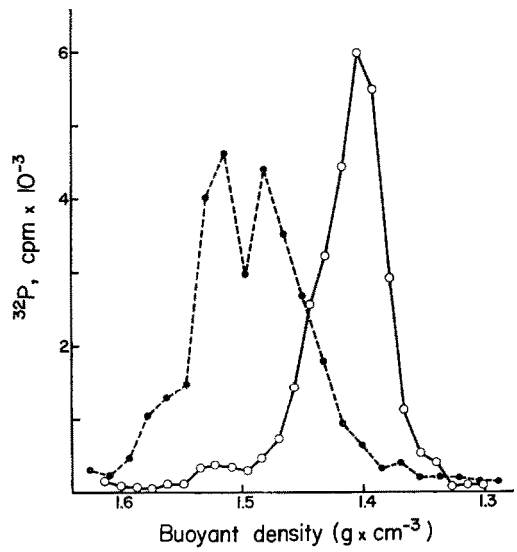

Fig. 7. Effect of EDTA on the buoyant densities of the ribosomal mRNP complexes containing *in vitro*-synthesized SFV RNA. The crude enzyme-template complex was incubated at 28° C for 30 minutes in the assay mixture containing ^{32}P-GTP. The reaction product present in supernatant 1 was then subjected to a sucrose gradient centrifugation analysis identical to that shown in Figure 5. The 100S to 160S region was pooled, divided into two parts, diluted 4× with RSB, and pelleted in a SW41 rotor for 12 hours at 190,000×*g*. One half of the pelleted polysomes was resuspended in RSB and served as a control; the other half was resuspended in TNE containing 30 mM EDTA. Each sample was then fixed in glutaraldehyde and analyzed on replicate CsCl gradients as described in the "Materials and Methods" section. Control (•); ribosomal mRNPs treated with EDTA (○)

The labeled structure sedimenting between 45S and 65S which had accumulated in the presence of puromycin was shown to be a ^{35}S-met-tRNA$_f$-40S-mRNP complex containing *in vitro*-synthesized 26S mRNA (unpublished data). When the triphenylmethane dye, aurintricarboxylic acid was added at the beginning of the *in vitro* reaction, this complex was not formed and the newly-synthesized ribosomal-free mRNPs accumulated as particles with buoyant densities in CsCl ranging from 1.32 to 1.40 gcm^{-3} (data not shown).

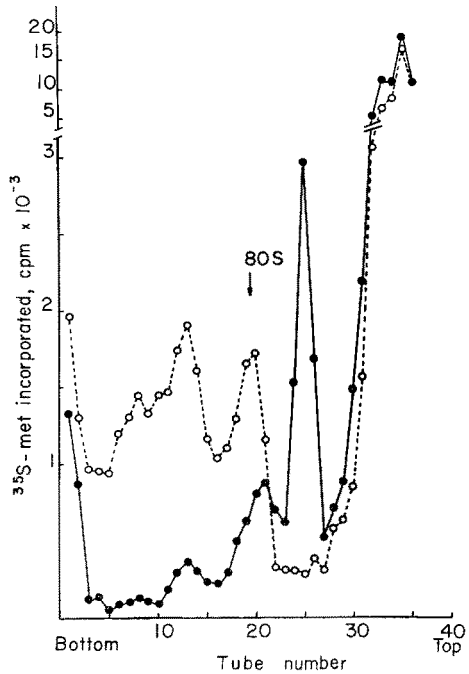

Fig. 8. Displacement of nascent polypeptide chains from polysomes by puromycin. The crude enzyme-template complex was divided in half. One half was incubated for 30 minutes in the presence of ^{35}S-met as described in Figure 4. The other half was incubated in the presence of ^{35}S-met alone for 25 minutes and then combined with 200 μg of puromycin per ml for an additional 5 minutes. Supernatants 1 of the control and puromycin treated extracts were analyzed on replicate sucrose gradients in RSB. Centrifugation was at 4° C for 14 hours in a SW 27 rotor at 38,000 × *g*. Fractions were collected, and absorbancy at 260 nm and acid precipitable radioactivity were determined as described in the "Materials and Methods" section. 80S ribosomes served as sedimentation markers. Control (○); puromycin treated (●)

Release of RNA From Complexes by Puromycin-High Salt

As a further demonstration that the *in vitro*-synthesized RNAs are incorporated into translational complexes, the rapidly-sedimenting structures were collected and treated with puromycin and high salt, a treatment known to dissociate actively translating polysomes into RNA and 60S and 40S ribosomal subunits (4, 6). Figure 9 shows that the ^{32}P-labeled complexes sedimented i) as a small peak near 40S and ii) as a major peak slower than 40S. Size class i) contained mostly

42S and some 26S RNA, whereas size class ii) contained 26S RNA only (data not shown). When the sedimentation profile of puromycin-high salt treated polysomes was analyzed separately, there was virtually no RNA found in structures sedimenting in the polysomal region.

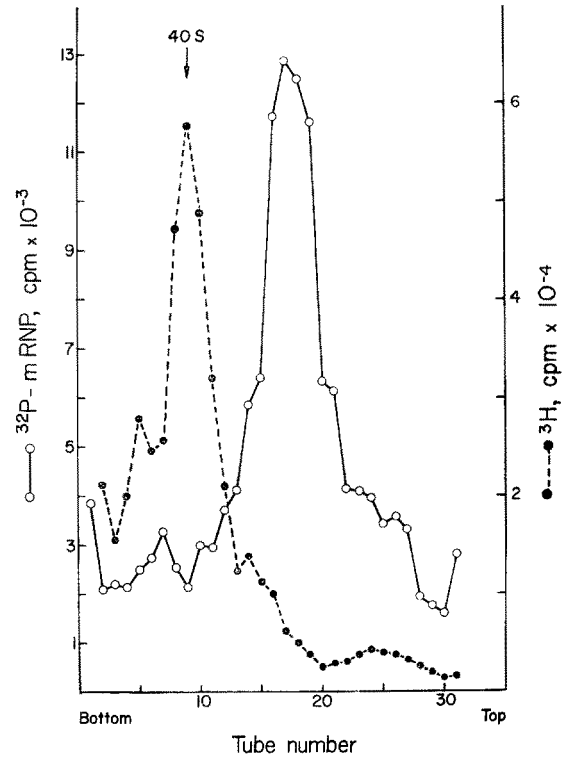

Fig. 9. Isolation of ribosomal-free mRNPs containing *in vitro*-synthesized 42S and 26S SFV RNA. The crude enzyme-template complex (see "Materials and Methods" section) was incubated at 28° C for 30 minutes in the assay mixture containing ^{32}P-GTP. Supernatant 1 was subjected to a discontinuous sucrose gradient centrifugation analysis containing two layers of 1.6 M and 2.1 M sucrose, respectively, and the polysomes were pelleted at 190,000 × *g* for 18 hours in a SW 41 rotor. The polysomes were resuspended in 1 mM puromycin—0.5 M KCl buffer and incubated for 10 minutes at 0° C, followed by 10 minutes at 37° C. The puromycin treated polysomes were layered onto a 5 to 20 per cent sucrose gradient in high salt buffer. The gradient was centrifuged in a SW 41 rotor at 22° C for 3 hours at 190,000 × *g*. Fractions of 0.35 ml were collected, acid-precipitable radioactivity and absorbancy at 260 nm were determined as described in the "Materials and Methods" section. The 40S ribosomal subunits served as sedimentation marker

Discussion

In an extract derived from SFV infected cells, the *in vitro*-synthesized 42S and 26S RNAs are present in structures whose sedimentation coefficients are higher than that expected for free, deproteinized RNA. On the basis of their physico-chemical properties at least some of the structures can be defined as free

mRNPs and as ribosomal initiation and elongation complexes. The newly-synthesized 42S and 26S RNAs are found in two distinct states: i) associated with membranes of large size, most of which are pelleted at $250 \times g$ in 10 minutes; and ii) as non-membrane bound mRNPs that are either free or bound to the 40S ribosomal subunits or to ribosomes. The non-membrane bound mRNPs are collected in the supernatant during low-speed centrifugation. When the pelleted, large cytoplasmic membranes are treated subsequently with Tween 40 and DOC under conditions known to leave mRNPs intact, a large portion of the membrane-bound mRNPs containing the newly-synthesized 26S RNA is dissociated as free mRNPs, sedimenting at about 33S. The finding that the membrane-associated 26S is solubilized only when the rapidly sedimenting structures are treated with Tween 40-DOC, indicates that it is tightly bound to lipoprotein membranes. When subjected to Cs_2SO_4-DMSO gradients, the 33S complex bands at an average buoyant density of 1.50 gcm^{-3}. Since free RNA bands at 1.63 gcm^{-3} and mRNPs dissociated by EDTA in low salt band at 1.38 gcm^{-3} in these gradients (25), this complex contains considerably less protein than free mRNPs. The protein(s) present in the 33S particle appears to have a high affinity for the 26S RNA, since the complex remained stable during centrifugation in Cs_2SO_4 gradients when fixation in glutaraldehyde was omitted. The characterization of the 33S particle and its fate during a continued *in vitro* incubation is the subject of a further investigation to be described in a future report.

In contrast to the membrane-bound 26S RNA, the non-membrane bound mRNPs are collected in the supernatant after low-speed centrifugation and are the main subject of this study. The newly-synthesized 42S RNA is found in structures with sedimentation coefficients ranging between 100S and 160S, whereas the 26S RNA sediments with structures from 30S to 160S (Fig. 2). Identical sedimentation patterns were obtained when the non-membrane bound structures were treated with Tween 40 and DOC prior to their sedimentation analysis. This indicates that the increased sedimentation coefficients of the 42S and 26S RNA in these structures is not due to a fortuitous association with lipoprotein membranes of small size. The mRNPs sedimenting between 30S and 160S show a considerable heterogeneity in CsCl gradients. Their buoyant density distribution corresponds to that reported for free mRNPs and mRNPs associated with ribosomal subunits and ribosomes (17, 21, 32, 33, 37).

It was reported that the 42S SFV RNA, when added to a cell-free *in vitro* protein synthesizing system, is found in ribosomal initiation and elongation complexes sedimenting between 150S to 300S (34). The present study shows that after a 30-minutes reaction approximately 40 per cent of the total *in vitro*-synthesized 42S RNA sediments as non-membrane bound mRNP complexes between 100S to 160S. On the basis of their buoyant density in CsCl and their sensitivity toward EDTA and puromycin, most of these structures appear to represent ribosomal initiation complexes. However, the possibility can not be excluded that at least some of the non-membrane bound 140S to 160S RNPs represent viral cores with a reported buoyant density in CsCl of 1.43 gcm^{-3} (42) (see also Figs. 1A and 5A and B, respectively).

The extract obtained from SFV infected cells allows the participation of added $40S_n$ particles with *in vitro*-synthesized 26S and 42S RNA in the assembly of

ribosomal initiation and elongation complexes. There exists a precursor-product relationship between the added $40S_n$ particles and ribosomal initiation and elongation complexes: When $40S_n$ particles were added to the reaction mixture and their fate followed during the next 15 minutes of incubation, about 50 per cent were chased into structures with sedimentation coefficients of 60S and higher (Figs. 4 and 5). With increased sedimentation coefficients of the complexes containing the added $40S_n$ particle a concomitant increase in their buoyant density was observed. Thus, the $40S_n$ particles underwent several *in vitro* transitions relative to their sedimentation coefficients as well as to their buoyant densities presumably reflecting the different stages involved in the formation of translational initiation and elongation complexes. When ^{35}S-met was added in the presence of $40S_n$ particles to the reaction mixture, the ^{35}S-label was found predominantly in structures with sedimentation coefficients of 60S and greater (Fig. 4B). Significant amounts of ^{35}S-met-tRNA$_f$ from these structures were not recovered. That these complexes were apparently involved in elongation of protein synthesis was demonstrated by the formation of ^{35}S-methionyl-puromycin when puromycin was present in the reaction mixture. The relative moderate chargeability of the added $40S_n$ particles with ^{35}S-met-tRNA$_f$ may be attributable to the fact that between 60 to 80 per cent of the native 40S subunits carry already a tRNA (43). It has been also reported that during centrifugation through sucrose gradients a significant amount of bound met-tRNA$_f$ is detached from the $40S_n$ particles (41). This finding is supported by the demonstration in this study of free ^{35}S-labeled met-tRNA$_f$ which is present on top of our sucrose gradients (Fig. 4A and B).

That most of the structures sedimenting between 100S and 160S were polysomes was shown by their sensitivity toward EDTA and puromycin. EDTA disrupts polysomes such that virtually all of the 26S RNA present on polysomes is found in a 65S particle (28) which bands in CsCl between 1.40 and 1.43 gcm^{-3} (Fig. 7). Using oligo(dT)-cellulose chromatography for the isolation of the 65S mRNP, we have shown that this particle still contains large amounts of 40S ribosomal proteins and 18S ribosomal RNA suggesting the presence of the smaller subunit (M. R. MICHEL, unpublished observations). In contrast, the puromycin-high salt released a mRNP sedimenting slower than 40S (Fig. 9) which is devoid of any ribosomal protein or ribosomal RNA. The involvement of the 100S to 160S structures in protein synthesis was further demonstrated by the displacement of initiation peptides and nascent peptide chains by puromycin.

Using oligo(dT)-cellulose chromatography for the isolation of free mRNPs, we have shown that about 80 per cent of the *in vitro*-synthesized 42S and 26S SFV RNA present in these mRNPs had poly(A) tracts (29). The strong association of the RNA and protein present in these mRNPs was affected neither by high salt (0.5 M KCl) nor by formamide, excluding the possibility that the free mRNPs are non-specific RNA-protein aggregates (3). The characterization of the proteins present on the high-salt resistant mRNPs and their topographical assignment are presently investigated in this laboratory.

The 26S RNA of Sindbis virus and SFV contains the genetic information for all the structural polypeptides of the virus (8, 11). Initiation of protein synthesis takes place at a single site on the 26S mRNA (9, 12) and the gene for the viral

core protein is the first to be translated (10, 22). At this stage the 26S mRNP-elongation complex is not yet membrane-associated (45) and the core protein is cleaved from polysomes before the entire 26S RNA is translated (10, 22). It has been suggested that the amino termini of the nascent envelope proteins coded for by the 26S RNA direct the binding of the elongation complex to membranes of the endoplasmic reticulum (45). For this reason, in virus-infected cells a large percentage of the polysomes containing the 26S RNA are membrane-bound (27, 45).

The present study shows that in contrast to the *in vivo*-synthesized 26S mRNA the majority of the *in vitro*-synthesized 26S ribosomal mRNPs in our crude enzyme-template complex are not bound to membranes. This is in agreement with the findings that the synthesis of the core protein is a membrane-dissociated event (45). Since we find most of the initiation and elongation complexes not bound to membranes, one has to assume that during the 30-minutes reaction elongation beyond the first gene product is a rare event in our *in vitro* system. Although it was conceived for optimal synthesis of SFV RNAs, this system in addition closely imitates the *in vivo* situation in regard to the initial stages of the formation of initiation and elongation complexes.

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