

## Formation of Sindbis Virus Capsid Protein in Mammalian Cell-Free Extracts Programmed with Viral Messenger RNA

(reticulocyte/ascites/polycistronic/sodium dodecyl sulfate-gels/baby hamster kidney (BHK) cells)

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**ABSTRACT** Extracts from Krebs II ascites cells and rabbit reticulocytes effectively synthesize viral proteins with Sindbis viral mRNA isolated from Sindbis-infected BHK cells. The major product is identical to Sindbis capsid protein on the basis of its electrophoretic mobility in sodium dodecyl sulfate-acrylamide gels and two-dimensional tryptic-peptide fingerprints. Various amounts of several additional discrete polypeptides are formed, depending on the components of the cell-free extracts. One of these polypeptides may be a prematurely terminated part of the viral-capsid protein, while another is larger in molecular weight than capsid protein but contains the capsid tryptic peptides. Several of the proteins formed *in vitro* also are detected in extracts of Sindbis-infected BHK cells labeled with [<sup>35</sup>S]methionine.

The three proteins found in Sindbis virions are postulated to originate by proteolytic cleavage from a larger molecular weight polypeptide precursor that is translated from a polycistronic mRNA presumed to contain a single site for initiation of protein synthesis. The two *in vitro* systems appear to translate this polycistronic viral mRNA to yield specific viral capsid although no evidence was found for post-translational proteolysis. Other mechanisms for production of the capsid protein in the cell-free extracts are considered, and some of these may function in the viral-infected cell where unusually large amounts of viral capsid proteins are frequently detected.

*In vitro* synthesis of proteins directed by mRNAs derived from animal viruses can provide important information about the processing of viral proteins and the role of various RNA species observed in virions and viral-infected cells. There are now a number of examples in which viral-specific RNAs act as messenger RNAs in cell-free systems, and several of these are reported to make proteins closely related or identical to viral proteins isolated from viral-infected cells. The polycistronic RNA from encephalomyocarditis virus makes a large polypeptide *in vitro* that resembles the precursor polypeptide known to be cleaved in a cascade of post-translation proteolysis that leads to the formation of the specific virion proteins (1-7). In contrast, the monocistronic mRNAs isolated from reovirus-infected cells direct the synthesis of eight polypeptides in a reticulocyte cell-free system that are indistinguishable from the proteins formed *in vivo* (8,9). More recently, mRNA isolated from cells infected with a paramyxovirus has been shown to generate virion capsid protein *in vitro* (10).

In the work presented, we have examined two mammalian cell-free systems for their ability to translate RNA from Sindbis virions and Sindbis-infected BHK cells. Sindbis is a relatively simple enveloped virus with a RNA genome of molecular weight about  $4 \times 10^6$  and sedimentation coefficient of 42-49 S (11, 12). Infection of BHK cells by Sindbis shuts

off host protein synthesis and leads to the predominant appearance of a 26S RNA that is believed to function as a polycistronic mRNA for the virion proteins (11-14), in a manner analogous to that demonstrated for the picornaviruses. Presumably, the balance of the genome codes for viral-specific proteins and may function as monocistronic mRNAs. An *in vitro* protein-synthesizing system should aid in elucidating the mechanisms by which Sindbis viral proteins are formed. This report describes our initial results with cell-free extracts incubated with Sindbis virus mRNA and shows that the major polypeptide formed *in vitro* is identical to the virus capsid protein.

### MATERIALS AND METHODS

**Preparation of Sindbis Virion RNA.** Virus was grown in monolayers of BHK21 cells, and plaqued on chicken-embryo fibroblasts as described (15), and purified by precipitation with 10% polyethylene glycol (16) followed by centrifugation for 12-16 hr through a composite velocity and equilibrium gradient (17). Virus was collected from the gradient and centrifuged for 2 hr at  $100,000 \times g$ . RNA was recovered from the pellet of virus by extraction with phenol-chloroform mixture (18) or by a modification of the method of Kirby (19). RNA was further purified on a 10-20% sucrose gradient containing 0.1 M NaCl, 50 mM Tris·Cl (pH 7.4), 1 mM EDTA, 0.5% sodium dodecyl sulfate.

**Preparation of Sindbis Viral mRNA.** Viral-specific mRNA was labeled by addition of [<sup>3</sup>H]adenosine to monolayers of BHK21 cells 1 hr after cells had been infected with Sindbis virus and given 1 μg/ml of actinomycin D. Nine hours later the cells (about  $4 \times 10^8$ ) were lysed with 60-80 ml of a solution containing 1% Triton X-100, 10 mM NaCl, 10 mM Tris·Cl (pH 7.4), 1.5 mM MgCl<sub>2</sub>, and 10 μg/ml of polyvinyl sulfate. Nuclei and cell debris were removed by brief centrifugation, and cytoplasmic RNA was extracted with phenol-chloroform mixture. The aqueous phase was precipitated with cold ethanol, and the pellet was dissolved in H<sub>2</sub>O and made 2 M in LiCl. The precipitated material (single-stranded RNA) was suspended in about 5 ml of a buffer containing 10 mM Tris·Cl (pH 7.5) and 0.5 M KCl. This solution of RNA (100-150 A<sub>260</sub> units) was applied to a small column (5 ml total volume) of oligo(dT)-cellulose (Collaborative Research, Inc., Waltham, Mass.) (20, 21). Bound RNA was eluted at a flow rate of 6 ml/hr with 10 mM Tris·Cl (pH 7.4) and collected in sterile tubes. Those fractions with A<sub>260</sub> and/or <sup>3</sup>H counts were pooled and precipitated with ethanol. This fraction is referred to as viral mRNA. Approximately 10-20%

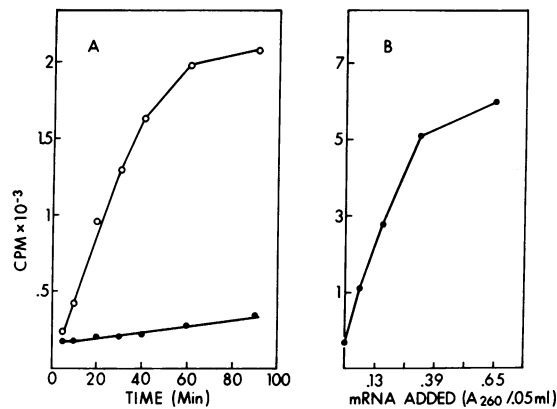


FIG. 1. Protein synthesis in ascites cell extracts provided with viral mRNA. (A) (●—●) No added RNA; (○—○) with 16  $\mu\text{g}$  of RNA. (B) Incorporation measured after 90 min of incubation at 29°. Refer to *Methods* for additional details. Total volume of reaction mixtures was 0.06 ml. Values for cpm have been multiplied by  $10^{-3}$ .

of the [<sup>3</sup>H]RNA added to the column was recovered in this mRNA fraction. An analysis of the mRNA fraction by electrophoresis in gels containing 1.8% acrylamide and 0.5% agarose revealed peaks of [<sup>3</sup>H]RNA that corresponded to size classes of 33 S, 24–26 S, and 18–20 S. The latter represented a minor percentage (<5%) of the added [<sup>3</sup>H]RNA, and the two larger size classes varied in relative amounts in the different preparations.

**Preparation of Cell-Free Extracts.** Rabbit reticulocytes were obtained from rabbits made anemic with phenylhydrazine according to the method of Adamson *et al.* (22). One volume of washed rabbit reticulocytes was lysed with one volume of cold distilled H<sub>2</sub>O and centrifuged at 20,000  $\times g$  for 20 min. The supernatant fraction (called S-30) was used at a concentration of 0.5 ml/ml of incubation mixture (CFSI). For CFSII, lysed reticulocytes prepared as above were centrifuged for 2 hr at 100,000  $\times g$ . The supernatant fraction (called S-100) was used without further fractionation. To the pellet fraction was added a solution of 25% sucrose in 0.5 M KCl so that the final concentration of ribosomes was 20–25 mg/ml, assuming that 1 mg of ribosomes per ml gives an  $A_{260}$  of 11.2 (22). This solution was resuspended with a Dounce homogenizer, incubated 4° for 30 min, and centrifuged 2 hr at 100,000  $\times g$ . The supernatant fraction was referred to as a salt-ribosomal wash. The pellet was suspended in a small volume of 10 mM Tris·Cl (7.4)–10 mM NaCl–15 mM MgCl<sub>2</sub> and represented the salt-treated ribosomes.

The S-30 cell-free extracts from Krebs II ascites cells contained 10–15 mg/ml of protein. This extract and tRNA (60  $A_{260}$ /ml) from rat liver were generously provided by Dr. I. Boime, and were prepared as described by Mathews and Korner (1) and Aviv *et al.* (23).

**Composition of Reaction Mixtures for In Vitro Protein Synthesis.** (Unless noted otherwise, the amounts are for 1 ml of reaction mixture.)

(A) Ascites cell-free extract (3–4.5 mg of protein of S-30 preparation); 2.4  $A_{260}$  units of tRNA; 20 mM Tris·Cl (pH 7.4); 3 mM Mg acetate; 56 mM KCl; 7 mM 2-mercaptoethanol; 1 mM ATP; 0.1 mM GTP; 0.6 mM CTP; 10 mM creatine phosphate; 12 units of creatine kinase (EC 2.7.3.2); 19 nonradioactive amino acids at 0.1 the concentration noted

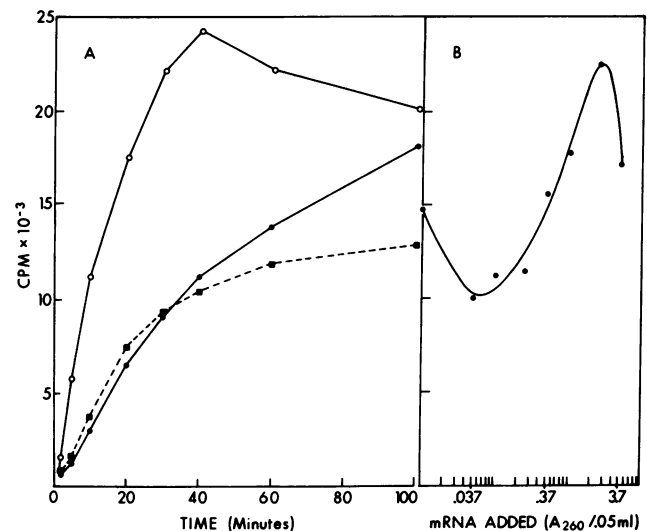


FIG. 2. Protein synthesis in extracts of rabbit reticulocytes (CFSII) provided with viral mRNA. (A) (●—●) No added RNA; (○—○) with 37  $\mu\text{g}$  of RNA; (■—■) with 0.7  $\mu\text{g}$  of RNA. (B) Incorporation after 90-min incubation at 29°. Refer to *Methods* for additional details.

by Borsook *et al.* (24), except that glutamate and aspartate were used at 40  $\mu\text{M}$ ; 5  $\mu\text{M}$  [<sup>35</sup>S]methionine, specific activity 4–40 Ci/mmmole.

(B) Reticulocyte cell-free extract (for CFSI, 0.5 ml of S-30 fraction from lysed reticulocytes; for CFSII, 0.5 ml of S-100 fraction plus 0.12 ml of salt-ribosomal wash plus 0.4 mg of salt-treated ribosomes); 40  $\mu\text{M}$  hemin; 2 mM Mg acetate; 80 mM KCl; 1 mM ATP; 0.2 mM GTP; 15 mM creatine phosphate; 15 units of creatine kinase; and nonradioactive amino acids and [<sup>35</sup>S]methionine as in (A).

**Isolation and Characterization of Translation Products.** Incubations were generally carried out at 29° for 90 min in volumes of 25–50  $\mu\text{l}$ . The reaction was stopped by the addition of an equal volume of a solution composed of 2% sodium dodecyl sulfate, 1 M Tris·Cl (pH 9.0), and 0.3 M 2-mercaptoethanol. The labeled proteins were alkylated by addition of iodoacetamide according to procedures described (25). Before electrophoresis they were dialyzed† against 0.1% sodium dodecyl sulfate, containing unlabeled methionine (1 mg/ml), and lyophilized. Samples were resuspended in a small volume of the electrophoresis buffer containing 10% glycerol, and 5- to 10- $\mu\text{l}$  samples were applied to slab gels containing 10% acrylamide and 0.1% sodium dodecyl sulfate. The amount of [<sup>35</sup>S]protein added to the gels varied from 5,000 to 50,000 cpm. In general, we added equal amounts of proteins from the reaction mixtures and varied the exposure times from 1 to 15 days to insure that all bands were visualized. Electrophoresis was at 120 V for about 2 hr in the apparatus described by Studier (26), and gels were prepared for autoradiography according to procedures described (25).

**Preparation of Viral-Specific Proteins from BHK Cells and Capsid Protein from Virions.** The methods for labeling Sindbis virus-infected BHK cells, isolation of labeled virions, and preparation of viral-specific proteins for sodium dodecyl

† A plastic form for dialyzing six 0.1-ml samples was designed and constructed by Mr. Richard McDonald of the Department of Microbiology, Washington University School of Medicine.

sulfate-acrylamide gel analyses were identical to those described (25).

**Preparation of Tryptic Peptide Fingerprints.** Proteins were eluted from preparative sodium dodecyl sulfate-polyacrylamide slab gels by cutting bands from the dried gel after exposure to film and shaking these strips of gels with water at 30° for 16 hr. Procedures for tryptic digestion, electrophoresis, and chromatography of the peptides, and autoradiography have been described (25). Between 50,000 and 100,000 cpm were used for a fingerprint.

## RESULTS

**Characteristics of the In Vitro System.** RNA from purified virions and RNA isolated from viral-infected BHK cells was effectively translated by extracts from Krebs II ascites cells or rabbit reticulocytes. In reaction mixtures containing ascites extracts, protein synthesis directed by added viral mRNA continued at a linear rate for 30–40 min at 29°, and by 90 min reached a level some 6- to 7-fold greater than that recorded in the absence of viral RNA (Fig. 1A). The amount of protein formed after a 90-min incubation increased linearly with increasing amounts of added viral mRNA, and maximum incorporation was achieved with a concentration of 350 µg/ml of RNA (Fig. 1B). The optimal concentration of Mg<sup>++</sup> for this system was between 2 and 3.5 mM. The optimal concentration of KCl for the system was 45–60 mM when virion RNA was used and 55–70 mM when viral mRNA was used.

Protein synthesis in the extracts of rabbit reticulocytes was also stimulated by the addition of viral mRNA, but this stimulation was critically dependent on the concentration of mRNA. At low concentrations of added viral RNA, there was an inhibition of the endogenous incorporation (Fig. 2A). When the amount of added RNA was increased several-fold, a stimulation of synthesis relative to the endogenous level occurred (Fig. 2B). Even at the higher level of RNA, however, the incorporation of [<sup>35</sup>S]methionine into protein stopped after 30–40 min, whereas endogenous synthesis continued for at least 100 min (Fig. 2A). At all levels of added mRNA, there was a preferential translation of the exogenous mRNA. The inhibition of endogenous synthesis and early shut-off of translation of exogenous mRNA has been noted by others, but its biochemical basis remains unknown (8).

**Identification of the Proteins Formed by Viral RNA.** The polypeptides formed in the two cell-free systems were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in slab gels. The patterns of radioactive bands revealed by autoradiograms were compared with those proteins formed *in vivo* in BHK cells infected with Sindbis virus. The most striking feature of the band pattern from the cell-free systems is the preferential synthesis of a protein that has an electrophoretic mobility identical to that of the viral capsid protein (Fig. 3 top). Other discrete polypeptides are frequently detected when viral RNAs are added to these *in vitro* systems but their amounts vary with the different incubation mixtures. Reactions containing lysed reticulocytes produced more of the lower molecular weight proteins (gel 2, Fig. 3 bottom). The use of a reticulocyte system composed of salt-washed ribosomes, initiation factors, and S-100 fractions led to formation of larger proteins (gel 4, Fig. 3 bottom). Patterns of proteins similar to those of Fig. 3 top and bottom were obtained when virion RNA was used instead of viral mRNA. In general, however, the relative amount of

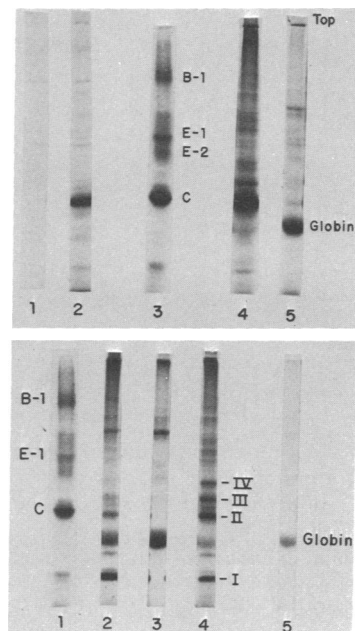


FIG. 3. (top) Sodium dodecyl sulfate-acrylamide gel patterns of viral proteins formed *in vivo* and *in vitro*. (1) No added RNA, ascites extracts; (2) with 20 µg of viral mRNA, ascites extracts; (3) protein synthesis in BHK cells 6 hr after infection with Sindbis virus; [<sup>35</sup>S]methionine was present for 60 min; B-1 is a precursor of the viral envelope proteins (E-1 and E-2), C is viral capsid protein; (4) with 22 µg of viral mRNA added to CSFII-reticulocyte preparation; (5) no added RNA to CFSII-reticulocyte preparation. (bottom) Comparison of viral protein synthesized by CFSI and CFSII reticulocyte extracts provided with viral mRNA. (1) viral proteins from Sindbis virus-infected BHK cells, 60-min labeling period; (2) CFSI preparation with 9 µg of RNA; (3) CFSI preparation with no added RNA; (4) CFSII preparation with 9 µg of RNA; (5) CFSII preparation with no added RNA. All reactions were in a total volume of 0.025 ml.

capsid (band II) was less and there were more of the larger molecular weight proteins.

The radioactive protein noted as band II in Fig. 3 bottom was isolated from preparative gels and digested with trypsin. The resulting tryptic peptides were analyzed by two-dimensional electrophoresis and chromatography, and autoradiograms were compared to that obtained from a fingerprint of authentic [<sup>35</sup>S]methionine-labeled virion capsid protein. It is clear from Fig. 4 that protein band II, having an electrophoretic mobility in sodium dodecyl sulfate gels identical to capsid, contains the same pattern of methionine tryptic peptides as detected in the capsid itself. A separate series of fingerprints of protein bands I, II, III, and IV showed almost identical patterns of peptides. All the capsid tryptic peptides appeared to be present in fingerprints of protein bands II, III, and IV, but the fingerprint of band I was clearly lacking at least one peptide (arrow in Fig. 4, left panel). Band I is estimated to be about half the molecular weight of band II, and we would have expected more methionine tryptic peptides to be absent. However, one or two other less well-resolved peptides might also be missing; alternatively, most of the methionine tryptic peptides may not be uniformly distributed in the capsid protein.

**Kinetics of Formation of Viral Proteins.** The fingerprints of the major polypeptides formed in these *in vitro* systems indicated that all contain capsid material. One (band I) had

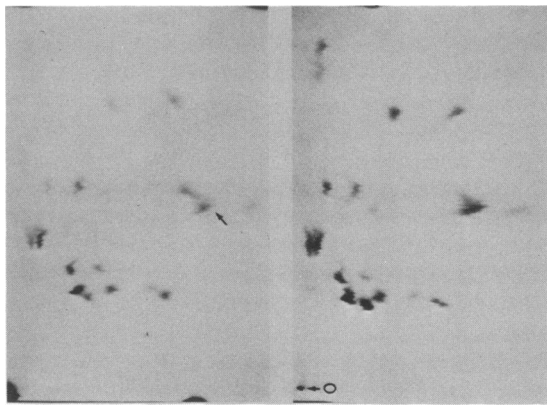


FIG. 4. Tryptic peptide fingerprints of capsid protein from virus and *in vitro* protein band II. Right pattern is capsid protein from purified virions; left pattern is from the *in vitro* protein band II (refer to Fig. 3, bottom), obtained from several reaction mixtures containing CFSII from reticulocytes and viral mRNA. "O" refers to site of sample application; electrophoresis was in the vertical dimension; chromatography was in the horizontal dimension. Arrow, peptide missing in band II, Fig. 3.

less than the full amount of the capsid protein, and the protein produced most frequently (band II) was authentic capsid. These data suggest that translation of the viral mRNA terminates at a few discrete places along the mRNA. To test this hypothesis, we examined the protein pattern in gels at 5, 10, 20, and 40 min after addition of mRNA to a reticulocyte cell-free system. The results are displayed in the autoradiogram of Fig. 5. The amount of radioactivity in bands I, II, III, and IV was analyzed by densitometry, and Fig. 5 (center panel) is a plot of the relative amounts of the individual polypeptides appearing as a function of time of incubation, normalized to the maximum amount of capsid that appeared at 20 min. Incorporation of isotope into protein by this cell-free system begins to shut off between 20 and 40 min (Fig. 5, left panel). The lower molecular weight species appears first (<5 min), followed by the capsid at 5 min and the longer polypeptides between 5 and 10 min. These data are consistent with a model in which translation initiates once on each of the added mRNA molecules and proceeds along a polycistronic RNA. At a few discrete points a portion of the RNA molecules seems to cease being translated and discrete polypeptides appear. In the particular reaction mixture described, there were about five times more capsid polypeptide formed than the other proteins. As shown in other autoradiograms (see Fig. 3 bottom), the capsid does not always appear in such disproportionate amounts. In most cases, however, we see a large amount of capsid and it may be that a special mechanism operates to release capsid (see Discussion).

#### DISCUSSION

We have demonstrated that cell-free extracts derived from Krebs II ascites cells and rabbit reticulocytes can translate Sindbis viral RNAs and that the major product is the virion capsid protein. These *in vitro* systems have been used extensively by several investigators for studying the translation of RNAs from a variety of animal viruses, and the proteins formed resemble the pattern of proteins detected in viral-infected cells (1, 3-6, 8-10). Protein bands I and II formed by the *in vitro* system described here are always found

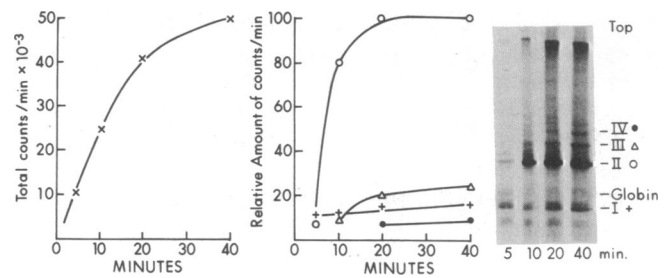


FIG. 5. Kinetics of *in vitro* synthesis of viral proteins. Reaction mixtures contained CSFII reticulocyte extracts provided with 12  $\mu$ g of viral mRNA in 0.05 ml total volume. Left panel: incorporation into trichloroacetic acid-precipitable material; center panel: relative amounts of labeled proteins in bands I through IV; right panel: autoradiogram of SDS-acrylamide gel electropherogram. The same amount of protein was added to each well of the slab gel.

in Sindbis virus-infected BHK cells. The larger molecular weight proteins (bands III and IV) are sometimes observed when infected cells are examined after very short pulses (2-5 min) of isotope.

The major methionine-labeled polypeptide detected in sodium dodecyl sulfate-polyacrylamide gels of an extract of Sindbis-infected BHK cells is the capsid protein, with the remaining label in the two envelope proteins of the virus and their immediate precursors. There is evidence that Sindbis virus capsid is formed from a polycistronic mRNA as the first polypeptide translated from the 5'-terminal region of the RNA and that the capsid is released as free protein before translation of the polycistronic RNA is completed (25). Release is believed to occur as a result of specific proteolytic cleavage, a mechanism that has been established for the processing of virion protein from larger precursor polypeptides in cells infected with picornaviruses (7, 27-31). With the picornaviruses, the data indicate that there are roughly equivalent amounts of all the cleavage products obtained from the larger precursor protein regardless of whether the smaller peptides came from the 5' or 3' end of the polycistronic mRNA. With Sindbis virus, however, there often appears to be about four to five times as much capsid produced *in vivo* than the other two proteins encoded by the polycistronic mRNA. The mechanism that can account for this asymmetric synthesis is unclear. Possibly the same mechanism operates in the cell-free translation system.

One explanation for the selective formation of capsid *in vitro* is a premature termination of the translation of the polycistronic mRNA occurring frequently at the carboxyl-terminal region of the capsid protein. Our *in vitro* kinetic data, together with the fingerprints of several polypeptides other than the capsid, suggest that these other polypeptides are formed as the result of termination at several discrete points along the mRNA. In this regard, our results resemble those reported by Boime and Leder (2) and by Kerr *et al.* (32), who studied the translation of encephalomyocarditis virus RNA in ascites cell-free extracts. Possibly these places on the mRNA are codons specific for rare species of tRNA and a stop or slow-down in translation allows for dissociation of the polyribosome complex and the cessation of further protein synthesis on that mRNA. Because the capsid sometimes appears five times more frequently in our *in vitro* preparations than these other fragments, it is conceivable that two of these rare codons occur in tandem at the end of

the capsid cistron. There is an interesting analogy to this kind of model; in bacterial mRNAs, the terminal "nonsense" codons appear to occur in tandem at the end of a cistron and they are responsible for termination of protein synthesis (33). Unlike the bacterial mRNAs, however, the polycistronic RNAs of the animal viruses appear to initiate translation only at the 5' end of the RNA; thus, termination of translation within the polycistronic mRNA from animal viruses would eliminate further expression of the mRNA.

One could also explain the enrichment in formation of capsid by assuming that preparations of mRNAs used in our systems contain a monocistronic fragment coding for capsid. We consider this an unlikely possibility because the viral mRNAs were of the order of  $10^6$  molecular weight on the basis of their electrophoretic pattern in acrylamide-agarose gels. However, the monocistronic fragment could be tightly associated to the large species or it could be generated in the reaction mixture by RNase action. Experiments are in progress to determine if a monocistronic RNA for capsid is generated in the reaction mixture or is part of the mRNA fraction.

We noted above that Sindbis virus capsid *in vivo* is believed to arise by proteolytic cleavage. We may be observing a similar mechanism in our *in vitro* studies, but we also consider this an unlikely explanation for the following reasons: (i) no differences in the sodium dodecyl sulfate-polyacrylamide gel protein patterns were detected when two general inhibitors of proteases were included in the reaction mixtures. We tested L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) at concentrations from  $10^{-5}$  to  $10^{-2}$  M and phenylmethyl sulfonyl fluoride from  $10^{-5}$  to  $10^{-3}$  M. Pfefferkorn has claimed that TPCK can inhibit the processing of Sindbis proteins *in vivo* (34). (ii) The ascites extracts we used were similar to those tested with encephalomyocarditis virus RNA, and little or no proteolytic cleavage was observed in those studies (2, 32). (iii) Assuming that protease action would not inhibit further translation, we should have detected additional polypeptides that did not contain capsid peptides; but no significant amounts of these were found. (iv) Data from the kinetic experiment (see Fig. 5) show that the larger peptides appear *after*, not *before*, capsid protein, a result inconsistent with a larger polypeptide as precursor of the capsid.

The cistron for capsid (molecular weight = 30,000) represents only about 10% of the genetic information in the viral genome and about one-fourth the coding information in the 26S RNA, which is the major species of viral RNA detected on polyribosomes in infected cells (11-14). Preliminary experiments indicate that 26S RNA, purified by electrophoresis in acrylamide-agarose gels, does form capsid protein *in vitro*. Although the Sindbis virus 26S RNA is presumed to function like the virion genomes of the picornaviruses as a polycistronic mRNA with a single initiation point, there are clearly other portions of the Sindbis virion genome whose expression is required for viral formation. These may appear as polycistronic mRNAs or as individual monocistronic messages such as those found in reovirus (35-37). Possibly our *in vitro* systems will be able to detect the additional viral-specific proteins and indicate whether they are encoded by mono- or polycistronic RNAs.

**Note added in proof.** Sindbis capsid is also the major protein found when purified 26S viral RNA is added to the CFS II reticulocyte system. In addition, viral RNA (26 S) from a tempera-

ture-sensitive mutant of Sindbis forms discrete capsid protein when incubated with CFS II at 29°, but at 39° (the nonpermissive temperature) capsid sequences appear in larger-molecular-weight polypeptides. Results similar to those presented in this paper have been found by Simmons and Strauss (*J. Mol. Biol.*, in press).

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