The Lack of Messenger Activity of Ribonucleic Acid Complementary to the Viral Ribonucleic Acid of Bacteriophage R17*

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SUMMARY

RNA complementary to bacteriophage R17 RNA, isolated from bacteria infected with the virus, failed to stimulate protein synthesis in cell-free extracts of Escherichia coli. Under conditions optimal for protein synthesis under the direction of R17 viral RNA, the incorporation of amino acids into peptide linkage in the presence of complementary strand RNA was the same as the incorporation in the absence of added RNA, and the products formed were indistinguishable from the endogenous products. With several preparations of complementary strand RNA a small amount of amino acid incorporation was observed, but it correlated with the degree of contamination by viral RNA. Furthermore, peptide mapping of the material formed revealed that viral coat protein was the major product. The lack of messenger activity of the complementary strand can be explained by the fact that it failed to bind to ribosomes. Although incapable of associating with ribosomes and of initiating protein synthesis on its own, complementary strand RNA enhanced the incorporation of amino acids directed by viral RNA. This stimulatory effect of complementary strand RNA is not understood. We have shown, however, that the complementary strand RNA became partially resistant to RNase after incubation with the viral RNA in the E. coli extracts, and that it associated with ribosomes if the viral RNA was present. The effects of complementary strand RNA on the messenger activity of R17 viral RNA and the increased protection against RNase degradation suggest, but do not prove, that an association of the two strands takes place under the incubation conditions for cell-free protein synthesis.

Double stranded bacteriophage RNA has been isolated from bacteria infected with bacteriophages (1-4). These double stranded forms are composed of viral RNA and the complementary or minus strand. A recent method for the isolation and purification of complementary strand RNA has made its characterization possible (5, 6). Isolated complementary strand RNA is not infectious, but it has been shown to serve as an efficient template for the synthesis in vitro of infectious viral or positive strand RNA (7).

In the present investigation we have explored the possibility that complementary strand RNA might act as messenger RNA. In cell-free extracts of Escherichia coli in which added viral RNA markedly enhanced protein synthesis (8-11), there was no indication that the complementary strand directed the incorporation of amino acids into peptide linkage. Although complementary strand RNA is in itself incapable of stimulating protein synthesis, it does appear to interact with the viral RNA to enhance incorporation directed by the viral RNA.

EXPERIMENTAL PROCEDURE

Bacterial Growth and Cell-free Extracts—E. coli strain Q13, a mutant isolated in Dr. W. Gilbert's laboratory, deficient in ribonuclease I, and possessing an altered polynucleotide phosphorylase (12-14), was kindly provided by Dr. A. Wahba, Department of Biochemistry, New York University School of Medicine. A fresh overnight culture (250 ml) was added to 25 liters of medium containing 1% tryptone (Difco), 0.1% yeast extract (Difco), 0.1% glucose, 0.8% sodium chloride, and 3.3 mM sodium phosphate, pH 7.0. The bacteria were grown at 35°
in a 30-liter fermentor (New Brunswick Scientific Company) with vigorous stirring and aeration. Harvesting of the cells by centrifugation (Sharples centrifuge) was started when the cell density reached about 1 x 10^8 per ml. During the 40-min harvesting period, stirring and aeration were continued in the fermentor, and the bacterial density increased between 2- and 3-fold. The bacteria were washed once with 0.01 M Tris-HCl buffer, pH 7.8, containing 11 mM magnesium acetate and 1 mM EDTA. The bacterial paste was then frozen as a thin film flattened in plastic bags between two cakes of solid carbon dioxide.

The frozen paste was fractured into small pieces which were placed in an Edlebo X-press (15, 16) previously chilled to -20°. After chilling the X-press for 20 additional min in a mixture of ethanol-water (6:4) and solid carbon dioxide, the cells were broken at a pressure of 28,000 psi. The broken cells were extracted following a modification of the procedures of Nirenberg and Matthaei (17), as previously described (9, 10), but without any preliminary incubation. After dialysis at 0° for 16 hours, the extract was frozen in small amounts and stored at -70°.

Labeled Chemicals and Measurement of Radioactivity—Uniformly labeled 14C-L-lysine (0.22 C per mmole), L-proline-3,4-H (5 C per mmole), and uniformly labeled 14C-UTP (0.57 C per mmole) were obtained from New England Nuclear. Uniformly labeled 3H-L-lysine (0.2 C per mmole) and 3H-histidine (3.4 C per mmole) were obtained from Schwarz BioResearch. The specific activities of amino acids used in some experiments are indicated in the figure legends.

Carrier-free 32P-orthophosphate was purchased from Tracerlab. Uridine-5-3H of specific activities between 20 and 25 C per mmole was purchased from Nuclear-Chicago.

In experiments with both 14C- and 3H-labeled amino acids, samples were mixed with 1 ml of 6% neutralized casein hydrolysate (Difco) containing 0.15 mg of bovine serum albumin. An equal volume of 10% trichloroacetic acid was then added rapidly. After the samples were heated at 90° for 15 min, they were filtered and washed on glass fiber pads with 5% trichloroacetic acid. Radioactivity in samples from incubations with 14C-labeled amino acids alone was determined on paper discs after treatment with trichloroacetic acid (18). Radioactivity in fractions from sucrose gradient centrifugations was measured on Millipore filters after precipitation and washing with 5% trichloroacetic acid containing 0.9% sodium pyrophosphate. Discs were counted in a toluene-based scintillator.

Various Types of Viral RNA—The RNA of bacteriophage R7 was labeled with 32P (19) and the virus was purified according to the method described by Vasquez, Granboulan, and Franklin (20). 32P-Labeled viral RNA was extracted from the purified virus with phenol (2). Double stranded RNA was labeled with 3H-uridine, purified, and denatured in dimethyl sulfoxide as described previously (21). This denatured RNA was then sedimented on a 5 to 20% sucrose gradient at 50,000 rpm for 165 min at 5° in the SW 50 rotor. The RNA sedimenting in the 27 S region of the gradient was collected, precipitated with 2 volumes of ethanol, and re-dissolved in 0.06 M KCl.

The complementary strand RNA was purified from unlabeled denatured double stranded RNA according to the method of Iglewski and Franklin (6). The purity of the final preparations of unlabeled complementary strand RNA used in the protein synthesis experiments was measured by the addition of a trace of intact R17 viral RNA, labeled with 3H-uridine, to the double stranded RNA following denaturation. The specific activity of the labeled viral RNA in the final purified preparation of complementary strand RNA was compared to the specific activity of the initial mixture of denatured double stranded RNA to determine the relative contamination of the preparation with viral RNA. Preparations of complementary strand RNA used were contaminated with from 4 to 10% viral RNA. Complementary strand RNA was also purified from denatured double stranded RNA that was labeled with 3H-uridine. The extent of contamination of these preparations with the viral RNA was not determined.

The concentrations of RNAs were estimated spectrophotometrically; an A260 of 23.3 was equivalent to 1 mg of RNA per ml (22).

Conditions of Incubation—Incubations were carried out at 35°. The incubation mixture contained 10 mM magnesium acetate, 3 mM ATP, 0.2 mM GTP, 10 mM phosphoenolpyruvate, 20 mM glutathione, 30 mM KCl, 20 mM 5-formyltetrahydrofolic acid (folinic acid; calcium leucovorin, Lederle), 50 mM Tris-HCl (pH 7.8), and the E. coli extract containing 3.5 mg total protein. For the 5-min incubations analyzed by sucrose gradient centrifugation no pyruvate kinase was added, and there was no preliminary period of incubation. All of the other incubations contained 30 µg of pyruvate kinase per ml (Boehringer Mannheim), and the components of the incubation were incubated for 10 min before the addition of the radioactive amino acid and template RNA. When labeled amino acids were used, all of the other 19 unlabeled L-amino acids were added to 100 µM. When no labeled amino acid was present, all 20 amino acids were used.

The concentrations of the viral or complementary strand RNA added to the reaction mixtures are given in legends to the appropriate figures and tables. A linear dependence of protein synthesis on the concentration of viral RNA added could be shown in the range between 0.1 and 0.5 mg per ml, as has been reported previously (8). In this range, the amounts of 30 S and 50 S ribosomal subunits diminished, and the 70 S and polysomal material increased with the increasing concentrations of added viral RNA; nevertheless, some ribosomal subunits were still detectable by sucrose gradient centrifugation when viral RNA was present at 0.8 mg per ml. Linearity below 0.1 mg per ml was difficult to show because of the relatively large background from endogenous incorporation.

The range of RNA concentrations used for the incorporation experiments was considerably higher than that for the binding studies. For the latter, labeled complementary strand was added at 0.4 µg per ml, and labeled viral RNA at 40 µg per ml. On the other hand, for the incorporation studies unlabeled complementary strand RNA was added at 0.15 to 0.2 mg per ml, denatured double stranded RNA at 0.8 mg per ml, and viral RNA from 0.05 to 0.8 mg per ml.

Analyses of Incubations—After incubation for 7 min, protein synthesis was stopped by the addition of 0.1 mg per ml of chloramphenicol. The 0.1-ml reaction mixture was chilled in an ice bath and then layered with a wide bore pipette onto a 10 to 40% sucrose gradient containing 0.06 M KCl, 0.01 M MgSO4, and 5 mM Tris-HCl (pH 7.4) buffer and centrifuged in the Spinco model L2-50 at 2°. Fractions from sucrose gradients were continuously monitored for absorbance at 260 nm.
on columns, 0.9 cm × 150 cm, of Sephadex G-75 (Pharmacia) in 50% aqueous acetic acid. Preparation of samples, trypic digestion, and the technique of peptide mapping involving two-dimensional electrophoresis have also been described previously (8).

RESULTS

**Lack of Messenger Activity in Complementary Strand RNA**—A number of preparations of complementary strand RNA were used in this study to test their ability to direct the incorporation of labeled amino acids into protein. In the example illustrated in Table I, the complementary strand RNA was contaminated with 4% viral RNA. This preparation did not significantly stimulate amino acid incorporation into acid-precipitable material, over and above endogenous incorporation (Table I). An increment of incorporation above endogenous protein synthesis was sometimes noted and was proportional to the contamination of a particular preparation with viral RNA.

The product formed in the presence of complementary strand RNA was indistinguishable from the endogenous product. A doubly labeled mixture of the product synthesized in an incubation containing complementary strand RNA with a product made under the direction of R17 RNA was subjected to gel filtration. The complementary strand RNA product was smaller than the bulk of the product made under the direction of viral RNA. As seen in Fig. 1, the complementary strand product emerged from the column along with lysozyme. This complementary strand product overlapped that tritium-labeled material in the R17 product which, in other double labeling experiments, was shown to be the endogenous product. It should also be noted that complementary strand RNA did not promote the incorporation of lysine into small peptides which might not have been precipitated by trichloracetic acid. As seen in Fig. 1, the elution patterns of both products were quite similar from the 50th fraction to the end of the elution profile. An indication of the size of the polypeptides eluting in this portion of the diagram is given by the position of lysozyme (mol wt 14,000) and glucagon (mol wt 3647).

The products synthesized in reaction mixtures in the presence of preparations of complementary strand RNA, known to be contaminated with greater amounts of the viral RNA, contained more of the higher molecular weight material that emerged from the column before lysozyme. Analysis of this larger material by peptide mapping indicated that it was identical with the product formed under the direction of the viral RNA itself, and consisted mainly of viral coat protein.

**Binding Studies with Complementary Strand RNA**—Tritiated complementary strand RNA was incubated for 7 min in the cell-free extract, and the reaction mixture was analyzed by sucrose gradient centrifugation. Under conditions in which a substantial proportion of 32P-labeled R17 viral RNA sedimented in the 70 S and heavier regions of the polysome profile (Fig. 2A), little radioactivity from the complementary strand preparation was associated with ribosomes (Fig. 2B, ○). The small portion of radioactivity from the complementary strand RNA preparation which was bound to ribosomes may have been due to contamination with R17 viral RNA. Most of the viral RNA, originally sedimenting at 27 S, remained in the 30 S region after the incubation. From our experiments it was not possible to ascertain whether any of this material was bound to the 30 S ribosomal subunit. Although complementary strand RNA did not bind to ribosomes on its own, some binding occurred that was dependent upon the addition of viral RNA. As seen in Fig. 2B (○), 2 to 3 times more complementary strand RNA was associated with ribosomes when a mixture of tritiated complementary strand RNA and an excess of 32P-labeled viral RNA were added together. The 32P radioactivity is not shown in Fig. 2B; its distribution in this double label experiment was essentially the same as that observed in Fig. 2A. Evidence presented below indicates that the binding of complementary
Fig. 2. Sedimentation patterns of R17 viral RNA and complementary strand RNA after incubation in extracts of E. coli. The RNAs were incubated for 7 min under the conditions for protein synthesis ("Experimental Procedure"); the 0.1-ml reaction mixtures contained 30 μg L-proline-3,4-3H (7.2 × 10⁴ cpm per nmole). One reaction mixture contained 0.3 mg per ml of R17 viral RNA (□—□); the other, 0.6 mg per ml of denatured double stranded RNA (■). A reaction mixture without added RNA was also centrifuged (○). Following the incubation, chloramphenicol was added and the reaction mixtures were layered on 10 to 40% sucrose gradients and centrifuged at 25,000 rpm for 9 hours in the SW 25.3 rotor at 2°.

Fig. 3. Sedimentation patterns of nascent protein synthesized under the direction of R17 RNA and of denatured double stranded RNA with extracts of E. coli. The RNAs were incubated for 7 min under the conditions for protein synthesis ("Experimental Procedure"); the 0.1-ml reaction mixtures contained 30 μg L-proline-3,4-3H (7.2 × 10⁴ cpm per nmole). One reaction mixture contained 0.3 mg per ml of R17 viral RNA (□—□); the other, 0.6 mg per ml of denatured double stranded RNA (■). A reaction mixture without added RNA was also centrifuged (○). Following the incubation, chloramphenicol was added and the reaction mixtures were layered on 10 to 40% sucrose gradients and centrifuged at 25,000 rpm for 9 hours in the SW 25.3 rotor at 2°.

As shown in Fig. 2B, a portion of the complementary strand RNA sediments at about 50 S when incubated with the viral RNA. Although not depicted in Fig. 2B, 32P-labeled viral RNA was also measured by double label scintillation counting. No corresponding 32P-labeled material was observed in the 50 S strand RNA to ribosomes is due to an association of the complementary strand with the viral RNA.

R17 viral RNA, complementary strand RNA, or a mixture of both was incubated in a volume of 0.1 ml with extracts of E. coli under the conditions for protein synthesis ("Experimental Procedure"). After 7 min, chloramphenicol was added to a final concentration of 0.1 mg per ml, and the reaction mixtures were layered on 10 to 40% sucrose gradients and centrifuged at 2°. A, the reaction mixture contained 4 μg of 32P-labeled R17 RNA (○) with a specific activity of 2.7 × 10⁴ cpm per μg. The absorbance at 260 μg was monitored continuously during collection of samples from the gradient (— —). The material was centrifuged in the SW 41 rotor at 40,000 rpm for 3.75 hours. B, the reaction mixtures contained 0.4 μg of 3H-uridine-labeled complementary strand RNA (specific activity 1.9 × 10⁵ cpm per μg) either in the presence (●) or in the absence (Curve B, ○) of 4 μg of 32P-labeled R17 viral RNA (the radioactivity of this RNA is not shown in the figure). Because of mechanical failure of the SW 41 rotor it was necessary to use the SW 25.3 rotor. Centrifugation was at 25,000 rpm for 9 hours.
The components of the reaction (see “Experimental Procedure”), in a 5-μl volume at twice their final concentration, were placed on a sheet of plastic (Parafilm, American Can Company, Neenah, Wisconsin) and mixed in a single droplet with an equal volume of 0.06 M KCl with and without 1.5 μg of the complementary strand RNA. This preparation of complementary RNA had an 8% contamination with viral RNA. The various amounts of R17 RNA, in a volume of 1 μl, were added to the droplet, which was then drawn up into three capillary tubes, each of 3-μl capacity (Drummond Scientific Company). These were sealed with a drop of mineral oil to prevent excessive evaporation. After 30 min, in a 35° incubator room, the contents of the capillaries were blown out onto paper discs and prepared for scintillation counting, as described under “Experimental Procedure.” The concentration of 14C-lysine was 22 μM, and 808 cpm in 3 μl corresponded to 1 n mole per ml of incubation (3 × 10^6 cpm per n mole).

<table>
<thead>
<tr>
<th>R17 RNA added</th>
<th>Lysine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without complementary RNA</td>
</tr>
<tr>
<td>mg/ml</td>
<td>nmoles/ml</td>
</tr>
<tr>
<td>None</td>
<td>0.8</td>
</tr>
<tr>
<td>0.05</td>
<td>0.9</td>
</tr>
<tr>
<td>0.10</td>
<td>1.5</td>
</tr>
<tr>
<td>0.20</td>
<td>3.0</td>
</tr>
</tbody>
</table>

**Table III**

*Association of RNase-resistant complementary strand RNA with ribosomes*

See text for explanation. Tritiated complementary strand RNA was incubated for 7 min under the conditions of protein synthesis, either alone or in the presence of 32P-labeled viral RNA as described in the legend to Fig. 2. The reaction mixtures were centrifuged at 40,000 rpm for 225 min at 2° on 10 to 40% sucrose gradients in the Spinco SW 41 rotor. The gradients were divided into three fractions indicated, with the absorbance of the bacterial extract at 260 μg as a guide. RNAs from these fractions were extracted with phenol (2). The total amount of radioactivity in each fraction is given in parentheses. The percentage of the counts which remained acid-precipitable after treatment at 37° with 1 μg per ml of RNase A for 10 min in 0.1 M NaCl, 1 mM EDTA, and 0.05 M Tris-HCl buffer (pH 7.2) is presented in the table. All of the radioactivity was acid-precipitable before treatment with RNase.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Ribonuclease-resistant RNA</th>
<th>Mixture of complementary RNA and viral RNA</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-Complementary strand RNA (alone)</td>
<td>#H-Complementary strand RNA</td>
<td>#P-Viral RNA</td>
</tr>
<tr>
<td>&gt;30S</td>
<td>19 (1279)</td>
<td>32 (3389)</td>
<td>0 (299)</td>
</tr>
<tr>
<td>30S</td>
<td>6 (836)</td>
<td>26 (695)</td>
<td>0 (1298)</td>
</tr>
<tr>
<td>&lt;30S</td>
<td>9 (3796)</td>
<td>8 (3713)</td>
<td>0 (3117)</td>
</tr>
</tbody>
</table>

About 10 times more viral RNA was added to the reaction mixture than complementary strand RNA. If the material sedimenting in the 50 S region contained viral RNA in equimolar proportion to the complementary strand RNA, it would not have been noticed since it would represent an undetectable amount of 32P.

Complexes of viral RNA and viral coat protein, i.e. partially encapsulated viral RNA (23, 24), would be expected to have sedimentation coefficients between 27 S and 84 S, the respective values for viral RNA and the virion. Thus the formation of a complex of complementary strand RNA with coat protein synthesized in vitro during the incubation could account for the material sedimenting in the 50 S region. However, the distribution of acid-precipitable #H-proline after a 7-min incubation in the extract in the presence of denatured double stranded RNA revealed no newly synthesized protein in the 50 S region of the gradient than after an incubation with the viral RNA alone (Fig. 3). It can be estimated from the amount of RNA sedimenting in the 50 S region (Fig. 2B) and from the specific radioactivity of the #H-proline that the binding of one newly formed protein subunit would have been represented by at least 1000 cpm. Therefore, it is unlikely that a protein-RNA complex could account for the 50 S peak. The nascent protein was, however, associated with ribosomes in the monomer, dimer, and trimer regions of the gradient, as expected from the distribution of the viral RNA (Fig. 2A).

A complex of viral RNA polymerase and its template RNA has a sedimentation constant of about 40 S (25). RNA polymerase, newly synthesized under the direction of viral RNA, might sediment with its template, the complementary strand. This explanation for the presence of complementary strand material in the 50 S region is also unlikely, since we did not observe newly synthesized protein in that region. Moreover, we tested directly whether a replication complex was formed in the extracts by incubating RNA under the conditions of protein synthesis with #H-UTP. The incorporation of UTP into RNA was about 0.04 μmole per ml, and was not stimulated by the addition of either viral RNA or denatured double stranded RNA. Incorporation also occurred in the presence of chloramphenicol at a concentration of 0.1 mg per ml, indicating that it did not depend upon protein synthesis in vitro. This slight incorporation of UTP was possibly due to some DNA-dependent RNA-polymerase in the extract.

**Association of Complementary Strand RNA with Viral RNA in Cell-free Extracts**—The presence of complementary strand RNA in incubations containing R17 RNA did not interfere with the messenger activity of the viral RNA. On the contrary, as shown in Table II, it was stimulatory. Because the supply of complementary strand RNA was limiting, these incubations were carried out in extremely small volumes and the results were not as reliable as our previous measurements. Nevertheless, the incorporation of lysine into protein depended upon the concentration of R17 RNA at concentrations greater than 0.1 mg per ml, both in the presence and in the absence of complementary strand preparations. Enhancement of the incorporation directed by the viral RNA was greatest at the lowest concentration of viral RNA and diminished as this concentration increased. This effect of complementary strand RNA on protein synthesis suggested an interaction between it and the viral strand.

A further indication of interaction between the two strands was obtained by testing the ribonuclease resistance of a mixture of tritiated complementary strand RNA and #P-labeled viral RNA after a 7-min incubation in extracts under the conditions for protein synthesis. The reaction mixture was centrifuged and the gradient was divided into three fractions: material
having a sedimentation coefficient greater than 30 S, 30 S material, and material sedimenting more slowly than 30 S. The fractions were extracted with phenol in order to eliminate protein which might interfere with digestion by ribonuclease A. The results of ribonuclease treatment of the three fractions are presented in Table III. The partial resistance to 1 μg per ml of ribonuclease A of the complementary strand RNA from the fractions of the gradient sedimenting at 30 S or faster than 30 S suggested that some association between the two strands occurred during incubation in the extracts, and that the complementary strand RNA found in the polysome region might be partially annealed with ribosome-bound viral RNA. The 32P-labeled viral RNA, which was measured in the same aliquots, appeared to be completely hydrolyzed by ribonuclease. Since the viral RNA was present at a much higher concentration than was the complementary strand RNA, any protection arising from annealing with the smaller amount of complementary strand RNA would not have been detectable.

Although we have presented evidence which suggests that partial association of the RNA strands did occur in extracts, complementary strand RNA did not associate with viral RNA in the absence of the reaction mixture. As shown in Table IV, denatured double stranded RNA did not become resistant to degradation by RNase during incubation at 37° in buffer of ionic strength 0.1 m or greater. Treatment of the mixture of nucleic acids with phenol did not enhance the association of the two strands. Partial resistance to RNase did occur, however, when the RNA was heated to 60°. The factors initiating the association in cell-free extracts are unknown, but there exists the possibility that an enzyme might catalyze the reaction at the low temperature of incubation.

Engelhardt, Robertson, and Zinder (4) have shown that the ratio of histidine to lysine incorporated into protein under the direction of R17 RNA and of denatured double stranded RNA. The concentration of viral RNA (triangles) was 0.3 mg per ml, and of denatured double stranded RNA (circles) was 0.6 mg per ml; 32P-labeled (Δ, ○) was present at a concentration of 20 μCi and 1420 cpm corresponded to 1 nmole per ml in the 20-μl samples (7.1 x 10⁶ cpm per nmole); 3H-histidine (Δ, ●) was present at 30 μCi and 16,000 tritium cpm corresponded to 1 nmole per ml (8 x 10⁶ cpm per nmole). The final volume of the reaction mixtures was 0.1 ml. The two isotopes were measured simultaneously by scintillation counting. The results of incubations in the absence of added RNA were subtracted; these are not shown, but amounted to 5 to 15% of the values obtained upon addition of the RNAs.

![Figure 4](http://www.jbc.org/content/244/2/741/F4.large.jpg)

**Figure 4.** The time dependence of 3H-histidine and 14C-lysine incorporation into protein under the direction of R17 RNA and of denatured double stranded RNA. The concentration of viral RNA (triangles) was 0.3 mg per ml, and of denatured double stranded RNA (circles) was 0.6 mg per ml; 32P-labeled (Δ, ○) was present at a concentration of 20 μCi and 1420 cpm corresponded to 1 nmole per ml in the 20-μl samples (7.1 x 10⁶ cpm per nmole); 3H-histidine (Δ, ●) was present at 30 μCi and 16,000 tritium cpm corresponded to 1 nmole per ml (8 x 10⁶ cpm per nmole). The final volume of the reaction mixtures was 0.1 ml. The two isotopes were measured simultaneously by scintillation counting. The results of incubations in the absence of added RNA were subtracted; these are not shown, but amounted to 5 to 15% of the values obtained upon addition of the RNAs.

**TABLE IV**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>RNA resistant to RNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Double stranded RNA</td>
<td>73.3</td>
</tr>
<tr>
<td>2. Double stranded RNA denatured in TE³0 containing 85.7% dimethyl sulfoxide</td>
<td>2.0</td>
</tr>
<tr>
<td>3. Denatured double stranded RNA incubated in STE³0 (37°, 15 min)</td>
<td>4.1</td>
</tr>
<tr>
<td>4. Denatured double stranded RNA incubated in STE³0 (0°, 15 min)</td>
<td>0.1</td>
</tr>
<tr>
<td>5. Same as Step 3, followed by one phenol extraction cycle</td>
<td>4.5</td>
</tr>
<tr>
<td>6. Same as Step 4, followed by one phenol extraction cycle</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* TE³0, 1 mM EDTA-0.05 M Tris-HCl buffer, pH 7.2.
* STE³0, 0.1 M NaCl-1 mM EDTA-0.05 M Tris-HCl buffer, pH 7.2.

**DISCUSSION**

A sufficient explanation for the failure of a species of RNA to function as a template during protein synthesis in vitro is its inability to bind to ribosomes. The feature in natural, as contrasted to synthetic, polynucleotides responsible for binding to bacterial ribosomes appears to be a particular sequence of
bases containing at least the codon for N-formylmethionine (26, 27). Furthermore, amino acid incorporation under the direction of RNAs which contain proper initiating sequences may be distinguished from the incorporation with polynucleotides which lack these sequences by their different patterns of dependence upon magnesium ion concentration, as discussed by Nakamoto and Kolakofsky (28). Incorporation with synthetic polynucleotides lacking initiating triplets is optimal at relatively high concentrations of magnesium ion; the translation of viral RNA, on the other hand, is optimal at lower concentrations (10, 29). The actual divalent ion concentrations used depend on the concentrations of other components included in the reaction mixture, particularly phosphorylated compounds (for example, phosphoenolpyruvate and ATP); under our conditions, optimal protein synthesis with viral RNA occurred at 10 mM magnesium ion concentration, and with synthetic polynucleotides between 15 and 20 mM. We have tested complementary strand RNA only at a magnesium ion concentration of 10 mM. Complementary strand RNA failed to stimulate the incorporation of amino acids into protein, and did not bind to ribosomes under conditions optimal for protein synthesis with R17 viral RNA. We feel that we have not missed incorporation because of the incorrect choice of divalent ion concentration, since appreciable incorporation occurs with synthetic polynucleotides even at this less than optimal magnesium ion concentration. Thus the initiator sequence would appear to be lacking or unavailable in the complementary strand RNA.

The negligible binding of radioactivity seen with the complementary strand preparation can be attributed to the presence of contaminating viral RNA. Our preparations of complementary strand RNA were contaminated with from 4 to 10% viral RNA, which do not bind to ribosomes to some extent. This contamination is, moreover, accentuated because the viral RNA carries complementary strand RNA into faster sedimenting material. Analysis, by sucrose gradient centrifugation, of the distribution of complementary strand RNA in cell-free extracts revealed that considerably more complementary strand RNA was present in the polysome region when the incubation was carried out in the presence of an excess of viral RNA, and also that complementary strand RNA appeared in the 50 S region. Although we have not been able to obtain evidence for the presence of any viral RNA in the 50 S region, the data do not eliminate the possibility that this finding is related to the association of complementary strand RNA with the viral RNA. Even in incubations in the absence of added viral RNA, the complementary strand RNA preparation displayed a slight, but reproducible, shoulder in the 50 S region. This may be another indication of the contamination of the preparation with viral RNA. The complementary strand material which sedimented in the 50 S region has not been further characterized.

Although the complementary strand did not possess messenger activity, its presence in extracts enhanced the efficiency of viral RNA in directing protein synthesis. This stimulatory effect of complementary strand RNA is not understood. Nevertheless, we have presented evidence for a direct interaction between the complementary strand and the viral RNA, for example, the partial resistance of the complementary strand to ribonuclease after incubation with viral RNA in cell-free extracts (Table III). In addition to the incompleteness of resistance, another indication that the two strands are not reannealed along their entire length is the observation that completely double stranded RNA has little or no messenger activity (4). The formation of a partially double stranded form would explain the appearance of complementary strand RNA bound to ribosomes, an appearance which was dependent upon the presence of the viral RNA.

The appearance of complementary strand RNA bound to ribosomes after incubation in the presence of R17 viral RNA may be an artifact. The concentration of the viral RNA used in the binding experiments was always considerably higher than that of complementary strand RNA. If the binding of RNA can be considered an equilibrium reaction (30) which is highly concentration-dependent, then it is possible that the concentration of complementary strand RNA alone was insufficient for binding, but that its binding to ribosomes was favored in the presence of the higher concentrations of the viral RNA. The binding complexes described here were detected after sucrose gradient centrifugation, and were therefore not in equilibrium with the free reactants. We are also prejudiced against a model for the binding reaction which involves non-specific mass action because of recent work (31, 32) on the mechanism of initiation of protein synthesis which elucidates the stepwise binding of messenger RNA and N-formylmethionyl RNA to the 30 S ribosomal subunit, followed by the addition of the 50 S subunit to form the functional 70 S messenger-ribosome initiation complex. We have not carried out detailed studies of the concentration dependence of the binding reaction upon either the viral RNA or the complementary strand beyond showing that the 30 S and 50 S ribosomal subunits were not limiting in the range of the RNA concentrations used.

Our results do not permit us to decide whether the association of complementary strand RNA with viral RNA observed in the cell-free extracts reflects a process which occurs during the multiplication of the bacteriophage in vivo. Studies on the synthesis of RNA in infected bacteria (33-35) have revealed uncharacterized species of RNA sedimenting in the 40 to 50 S region. Moreover, Engelhardt, Robertson, and Zinder (4) and Robertson, Webster, and Zinder (36) have suggested that the bulk of viral protein synthesized during infection is directed by a multistranded form of RNA (replicative intermediate). There is as much, if not more, replicative intermediate as single stranded viral RNA in polysomes at the end of the latent period or at later times in the viral growth cycle (35, 37). Thus the enhancement which we have observed in vitro may be an indication that partially double stranded RNA binds to ribosomes more efficiently than does the viral RNA alone. Alternatively, the association of viral RNA with the complementary strand RNA may alter the configuration of the viral RNA, which might then be translated more efficiently. The complex formed under our conditions, however, is quite different from the replicative intermediate used by Engelhardt et al. (4). We observed no change in the relative amounts of histidine and lysine incorporated in the presence of both complementary strand RNA and viral RNA, whereas Engelhardt et al. (4) have shown that the translation of viral RNA from the partially double stranded replicative intermediate is modified so that all of the product is viral coat protein.

Similar concentrations of viral RNA and complementary strand RNA were used in the experiments showing the enhance-

1 Similar results have been obtained by K. Iwawaki, M. Miller, A. Wahba, and S. Ochoa (personal communication).


3 W. Iglewski and R. M. Franklin, unpublished data.
ment of protein synthesis when both were present together (Table II; Fig. 4). Another explanation for the stimulatory effect of complementary strand RNA on protein synthesis would be a possible protection of viral RNA from destruction by nucleases. This interpretation may also be supported by the fact that stimulation was apparent only after 12 min of incubation (Fig 4). An enhancement of messenger activity in extracts of E. coli by heterologous RNA from rabbit reticulocytes was attributed to the protection of the autologous messenger RNA from destruction by nucleases (38).

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The Lack of Messenger Activity of Ribonucleic Acid Complementary to the 
Viral Ribonucleic Acid of Bacteriophage R17
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