IN VITRO TRANSLATION OF MULTISTRANDED RNA FROM ESCHERICHIA COLI INFECTED BY BACTERIOPHAGE f2

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Bacteriophages with RNA as their genetic material produce various structures which contain double-stranded RNA as intermediates in their replication.[1-5] The major distinction among these various intermediates seems to be the amount of single-stranded RNA associated with the double-stranded material. Most investigations have concentrated upon the role of these RNA's in phage replication, but recent observations[6,7] have shown that double-stranded RNA is associated with polysomes in infected cells during much of the replicative cycle.

It is evident that the control of protein synthesis during infection by RNA phages must involve factors other than the properties of the single-stranded viral RNA. This is true since, late in infection, RNA phage-infected cells synthesize a great majority of coat protein as compared to other gene products, while *in vitro* protein synthesis using free viral RNA does not lead to such an overwhelming majority of coat protein. Lodish and Zinder[4] have proposed that one element of this control might be that coat protein acts as a modulating factor by shutting off the synthesis of noncoat proteins, a hypothesis for which there is now some evidence.[8,9]

We have prepared structures containing double-stranded RNA from cells infected with the phage f2 and utilized such preparations as messengers in the *in vitro* amino acid-incorporating system. The only product obtained was phage-coat protein, suggesting that the structure of the multistranded intermediates themselves is probably an element involved in the control of protein synthesis late in infection.

Materials and Methods.—Bacteria and bacteriophage: S26 (K38) is a nonsuppressing strain of *Escherichia coli* K12. S26Rle (K37) contains the Su-I amber suppressor.[10] A19 is a mutant of *E. coli* Hfr-H with little RNase I activity.[11] The f2 is the wild-type RNA phage. Sus-3 is a mutant of f2 with an amber-suppressible mutation in its coat-protein gene.

Growth and purification of phage-specific multistranded RNA: The multistranded RNA was prepared by phenol extraction of phage-infected bacteria using a modification of the procedure of Franklin.[12] The bacteria (K38 or A19 for wild-type f2, K37 for mutants) were grown as described for the growth of large quantities of phage by Webster *et al.*[13] Infected complexes were harvested by rapid centrifugation through a Sharples continuous-flow centrifuge from 25 to 50 min after infection. The bacteria (about 75 gm wet weight) were broken in a BioTech Edebo press and suspended in 600 ml of MES-magnesium buffer (0.01 M 2-(N-morpholino) ethanesulfonic acid, 0.015 M magnesium sulfate, titrated to pH 6.0 with saturated Tris base). Two hundred ml of MES-magnesium-saturated phenol were then added to this solution, and the mixture was shaken vigorously for 6 min. The aqueous layer was then gently removed and extracted three more times. The RNA in the final aqueous layer was precipitated by storage overnight at $-20°C$ after the addition of 2 vol of cold absolute ethanol and potassium acetate to 1% w/v.

The precipitated RNA was resuspended in TSE buffer (0.05 M Tris-HCl, pH 6.85; 0.1 M NaCl; and 0.001 M EDTA). Double-stranded RNA was fractionated from the

bulk RNA by two passages through a column of cellulose CF-11, eluted with TSE containing 35% and 15% ethanol (v/v), and then TSE buffer alone.[12] No more than 10,000 OD_{260} units can be added per liter of column, and thus column sizes were scaled to this capacity.

The procedures described above were developed to purify in large amounts and as intact as possible the multistranded RNA structures found in phage-infected cells. Since any conclusions to be drawn using these materials are dependent upon our success in this endeavor, we have below discussed each step in the technique. The lysed cells were resuspended in MES-magnesium buffer because RNase III, an enzyme which specifically cleaves double-stranded RNA,[15, 16] will not function in this medium.[17] In addition, little of the cellular DNA appears to go into solution in this medium and ribosomes are preserved intact. The phenol step fractionates the soluble nucleic acids away from proteins and cell debris. The cellulose CF-11 column has been shown to separate RNA containing double-stranded regions from sRNA, larger single-stranded RNA, and DNA.[12] Specifically, 99% of the RNA isolated from purified f2 virus elutes in 15% ethanol, while double-stranded RNA (e.g., reovirus RNA) elutes quantitatively and repeatedly in TSE buffer alone.[8] If the 1.0% of the f2 RNA which elutes in TSE alone is rechromatographed, it elutes in 15% ethanol.[18] Franklin[12] reports similar experiments.

We conclude from these observations that nucleic acid molecules which elute in the third peak (TSE alone) of the cellulose CF-11 column upon repeated chromatography contain regions of double-stranded RNA. If single-stranded RNA elutes repeatedly from the third peak, it cannot be free but must be connected to regions of double-strandedness by stable linkages, as already concluded by Franklin.[14] Robertson et al.[16] have shown, by nuclease digestion, that single-stranded RNA will repeatedly elute in the third peak of the column while attached to double-stranded regions, but will revert to the second peak (15% ethanol) when these regions are digested away.

None of the above operations were designed to fractionate further the structures containing double-stranded RNA, and a mixture of purely and partially double-stranded RNA molecules is obtained, which we call "replicative ensemble" (RE). Precipitation in 1.0 *M* NaCl may be used in the isolation procedure immediately before cellulose chromatography, allowing separation on the basis of lengths of single strands attached to the double-stranded portion.[5, 12, 14] Molecules with larger amounts of single-strandedness (replicative intermediate, or RI) precipitate in concentrated NaCl, while those with smaller amounts (replicative form, or RF) remain soluble.

Growth and purification of C^{14}-labeled multistranded RNA: Bacteria were grown to 2×10^8/ml in D-O minimal salts[19] with glucose which had been supplemented with 2 μg/ml of guanine and adenine. The bacteria were centrifuged and resuspended in D-O glucose without adenine or guanine and infected at five phage/bacterium with f2. Twenty min after infection, C^{14}-labeled adenine and guanine (2 μc and 5 μg per ml) were added. At 40 min after infection the bacteria were centrifuged and resuspended in $1/_{15}$ vol of MES-magnesium buffer. One half of this volume of MES-magnesium-saturated phenol was added and the multistranded RNA was purified as described above.

Enzymatic digestions: RNase III[15, 16] digestions were performed at 2–5 units/mμmole of substrate polymer phosphorus. Digestions with pancreatic RNase were performed at an enzyme concentration of either 0.1 or 1 μg per ml at 35°C for 30 min. Digestions in high salt were performed in TSE and those in low salt were performed in no more than 0.1 × TSE. Pancreatic RNase digestion was monitored by the release of TCA-soluble radioactivity, or by sucrose density gradient centrifugation.

In vitro protein synthesis: The conditions for the incorporation of amino acids into phage-specific proteins in bacterial extracts supplemented with the phage RNA have been described by Webster et al.[13] and Engelhardt et al.[20]

The products were prepared for analysis on acrylamide gels and Sephadex G-75 according to the procedure described by Viñuela et al.[21] The acrylamide electrophoresis was kindly performed for us by Dr. Henri Fromageot after the method of Viñuela et al.[21] For analysis on Sephadex, the prepared product was layered onto a column of Sephadex

G-75 equilibrated with 0.01 M sodium phosphate, 0.15 M 2-mercaptoethanol, 1.0% sodium dodecyl sulfate, pH 7.2. The flow rate of the column was 4 ml/hr, and samples were assayed as described by Webster *et al.*[13]

Assay for the sus-3 hexapeptide: The hexapeptide (formyl-met-ala-ser-asn-phe-thr) produced by the sus-3 message is conveniently assayed using an adaptation, which was developed by Dr. Robert E. Webster, of previously published methods.[13, 20, 22–24] The assay takes advantage of three facts known about the hexapeptide, namely that it is uncharged at low pH, that it is soluble in 5% TCA, and that it is larger than amino acid species which are also uncharged at low pH. Radioactive phenylalanine incorporation directed by sus-3 RNA is terminated by chilling to 0°C. An equal volume of 10% TCA is added, and precipitation allowed to proceed for 15 min at 0°C. The precipitate is carefully removed by centrifugation and one-half ml of the supernatant is layered onto a column containing 75 × 7.5-mm Dowex AG 50 (200–400 mesh) topped with 140 × 7.5-mm of Sephadex G-10, both previously equilibrated with pH 1.9 buffer containing 87 ml of acetic acid and 25 ml of 88% formic acid per liter of water. The column is washed with the same buffer and twenty 1-ml samples are collected.

Two radioactive peaks elute from the column. The first is largely (70–80%) sus-3 hexapeptide, while the second is presumed to be radioactive phenylalanine which has been deaminated during the incorporation reaction. The amount of the sus-3 hexapeptide in the first peak can be further quantified by electrophoresis at pH 5.0 on cellulose acetate strips at 10 v/cm for 2 hr. The sus-3 hexapeptide migrates approximately 1–3 cm toward the anode and is easily distinguished from background material. The recovery of prepurified sus-3 hexapeptide after this two-step procedure is approximately 80%.

Results.—Characterization of RNA structures containing double-stranded regions: The RNA's prepared as described in *Materials and Methods* (RF, RI, and RE) have many of the properties of materials described in other studies.[1–3, 5, 12, 14, 25] Specifically, the RNA's can be shown to contain regions of single-strandedness by measuring their sensitivity to digestion with pancreatic RNase. At high salt concentrations this enzyme will digest single-stranded RNA, but not double-stranded RNA. In one preparation of RE, 21 per cent of the RNA was digested by pancreatic RNase in high salt, and 100 per cent in low salt. When sedimented on a sucrose gradient, it migrated as one broad band centering at about 16S. After digestion with pancreatic RNase in high salt, most of the material sedimented in a sharper peak at about 10–12S. Digestion in low salt left only material which remained at the top of the gradient. We conclude that a majority of the molecules contains regions of single-strandedness.

The amount of double-stranded RNA was measured in this same RE-RNA preparation using RNase III, the nuclease specific for double-stranded RNA. Digestions with this enzyme revealed 15–20 per cent of the RNA to be resistant to the nuclease. When the products of this digestion were analyzed by sedimentation through a sucrose gradient, they gave a broad peak of material 7S or lighter. This shows that each of the molecules must have contained some double-stranded RNA. When the products of this digestion were analyzed using a cellulose CF-11 column, they eluted in the second peak (single-stranded RNA) and in the first peak (TCA-soluble oligonucleotides and nucleotides). From these studies we conclude that RE contains double-stranded RNA (about 80%) and regions of attached single-stranded RNA (about 20%).

Studies similar to the above were carried out upon RF and RI prepared by

salt precipitation.   RI contains more single-stranded material than RE, while RF contains less, roughly in agreement with other studies.[3, 5, 12, 14, 25]   RF in our hands is not purely double-stranded, however, but contains some single-stranded regions by the above criteria.   Finally, no material resembling RE was obtained from uninfected cells.

Relative incorporation of amino acids into protein: RF, RI, and RE all stimulated incorporation of amino acids into protein in the *in vitro* system.   This messenger activity is due to the single-stranded RNA regions, and not to breakdown of the double-stranded RNA.   By varying the purification procedure (e.g., by passing the material under vacuum through narrow-bore pipettes) or by mild RNase treatment,[3] it is possible to obtain double-stranded RNA preparations with no single strands attached which do not stimulate protein synthesis.

Table 1 shows a comparison of the ability of single-stranded and various multistranded RNA preparations to stimulate protein synthesis as measured by the incorporation of C^{14}-lysine into TCA-insoluble material.   The ability of structures containing double strands to stimulate incorporation has been as high as 30 per cent that of single-stranded f2 for some preparations.   The lower level of incorporation per unit of RNA can be partially explained by the fact that the messenger segment of these molecules is only a fraction of the total RNA, and partially by the more severe conditions necessary to isolate the RNA's containing double strands.

Table 1 also shows incorporation directed by sus-3 RNA's.   Sus-3 contains an amber mutation at site 6 of the coat protein gene.[26]   RNA from sus-3 directs production of whole-coat protein molecules when the amber triplet is translated as a chain propagation signal (suppressing conditions), while it directs production of a hexapeptide under nonsuppressing conditions.[13]   This is reflected (Table 1) by the difference in the amount of C^{14}-lysine incorporated by the single-stranded sus-3 RNA under suppressing and nonsuppressing conditions.   There is also a twofold difference between incorporation stimulated by sus-3 RI and RF under these conditions (Table 1).   This fact indicates that this RNA stimulates the synthesis of coat protein in the same manner as does single-stranded RNA: from the beginning of the coat gene to the site of the amber triplet.

The assay for the sus-3 hexapeptide was utilized to confirm that sus-3 multistranded RNA was read properly.   Figure 1 shows the electrophoresis on cellulose acetate of hexapeptide-produced by sus-3 single-stranded and RF-RNA's. The peak, 1–3 cm from the origin, is absent from the background run, and also is not observed when wild-type RNA is used as message.   Similar results were

TABLE 1.   *In vitro protein synthesis by single-stranded and multistranded messenger RNA's.*

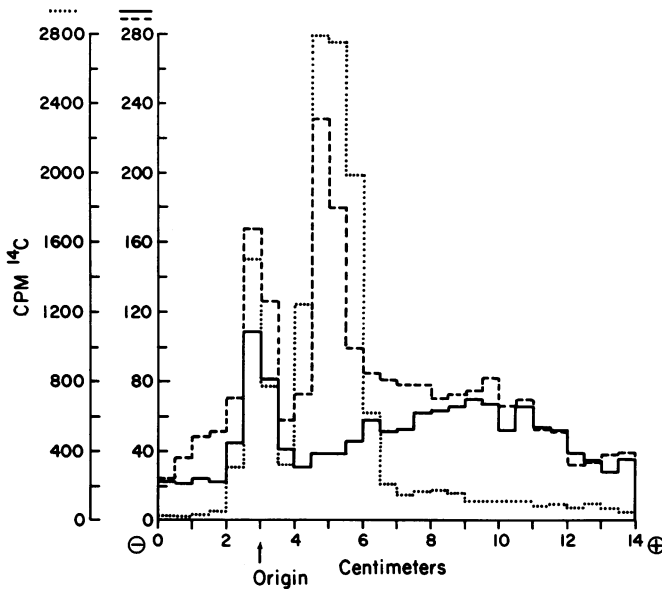| Messenger RNA	—Relative C^{14}-Lysine— Incorporation		Messenger RNA	—Relative C^{14}-Lysine— Incorporation	
	Suppressed	Not suppressed		Suppressed	Not suppressed
2 Single strands	100	100	f2 RE	12	12
Sus-3 single strands	65	15	Sus-3 RE	8	4

FIG. 1.—Electrophoretic assay using cellulose acetate strips of the amount of amino-terminal hexapeptide of coat protein stimulated by single- and multistranded sus-3 RNA.

The reaction mixtures (0.2 ml) were incubated 15 min at 35°C and then prepared for the electrophoresis as described in *Materials and Methods.*

The profiles represent product stimulated by: (· · · · · ·) 25γ sus-3 single-stranded RNA; (- - - - -) 90γ sus-3 RF-RNA; and (———) background incorporation without the addition of viral RNA.

In this run the hexapeptide is located from 1 to 3 cm to the right of the origin.

obtained with a preparation of sus-3 RI RNA. We calculate that at least two thirds of the "starts" stimulated by sus-3 RF under nonsuppressing conditions lead to the production of hexapeptide: a minimal estimate of the proportion of coat-specific starts. When suppressing sRNA is added to the incorporation mixture containing sus-3 RF, the amount of sus-3 hexapeptide is lowered by 50 per cent—a level comparable to suppression observed with sus-3 single strands.[22]

Analyses of coat versus noncoat products: Several techniques were employed to determine what products other than coat protein could be stimulated by f2 RE. The ratio of histidine to lysine incorporated gives an indication of the level of coat versus noncoat proteins stimulated by f2 RNA, since the coat protein contains no histidine.[20] Table 2 gives this ratio for various preparations of RNA. The major point illustrated is that in every case the amount of

TABLE 2. *His/lys ratios for single- and multistranded RNA's.*

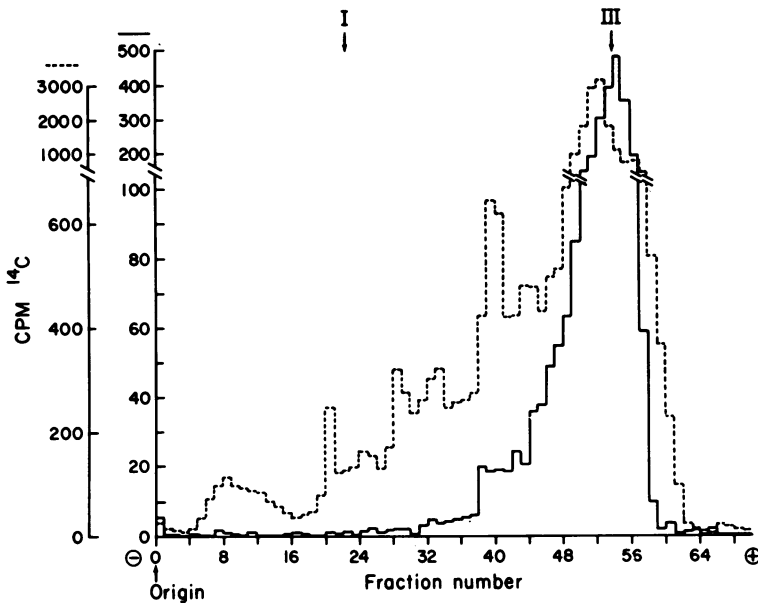| Messenger RNA	His/Lys Ratio		Messenger RNA	His/Lys Ratio	
	Suppressed	Not suppressed		Suppressed	Not suppressed
f2 Single strands	0.220	0.231	Sus-3 single strands	0.182	0.384
f2 RE	0.035	0.040	Sus-3 RI (or RF)	0.041	0.069

Fig. 2.—Polyacrylamide gel patterns of proteins directed by f2 and f2 RE.

The f2 single-stranded RNA and f2 RE were incubated in 0.2-ml reaction mixtures containing C^{14}-lysine for 15 min at 35°C. Both reaction mixtures were prepared for electrophoresis as described in *Materials and Methods*.

(----): Proteins directed by 75γ f2 single strands.

(———): Proteins directed by 300γ f2 RE.

"I" and "III" refer to the location of proteins synthesized by f2-infected spheroplasts. "III" corresponds to f2 coat protein.

histidine per unit lysine incorporated for the RNA's containing double strands is greatly reduced below that found for single-stranded RNA's.

Figure 2 shows the electrophoretic pattern on polyacrylamide gels of the proteins stimulated by f2 RNA and f2 RE. Figure 3 shows the elution profile from a column of Sephadex G-75 of the *in vitro* product stimulated by f2 single-stranded RNA and f2 RE. We conclude from these two experiments that, although single-stranded f2 RNA produces a variety of noncoat polypeptides, f2 RE makes nothing that runs differently from the coat protein (neither larger noncoat peptides nor peptides smaller than the coat protein).

Discussion.—The experiments described above show that structures containing double-stranded RNA purified from f2-infected cells are phage-specific and contain the coat gene in a translatable position, but are largely incapable of directing the synthesis of other virus-specific polypeptides. The exact nature of the structures responsible for the observed messenger activity—and particularly the identity of the free end present in RE-RNA—depends upon the mechanism of f2 RNA replication (see Fig. 4 for examples).

Regardless of the detailed mechanism of f2 RNA replication, the low level of noncoat protein synthesis which we have observed suggests a regulatory mechanism which may be operating *in vivo*. Lodish and Zinder[4] proposed that the

FIG. 3.—Sephadex G-75 patterns of proteins directed by f2 and f2 RE.

Single-stranded f2 RNA and f2 RE were incubated in 0.2 ml reaction mixtures containing C^{14}-tyrosine for 15 min at 35°C. Both reactions were prepared for chromatography as described in *Materials and Methods*.

(O - - - - O): Proteins directed by 56γ f2 single strands. (●————●): Proteins directed by 375 γ f2 RE.

Under these conditions, the excluded peak (as measured by bovine serum albumin) elutes at tube 24, while C^{14}-lysine elutes at tube 41. Endogenous protein synthesized when no messenger RNA is added elutes largely in the excluded volume, while coat protein from phage particles elutes in tubes 28 and 29.
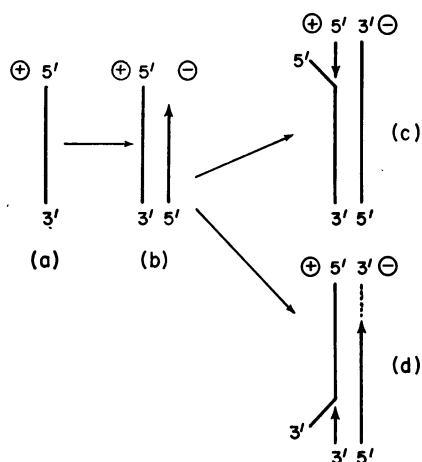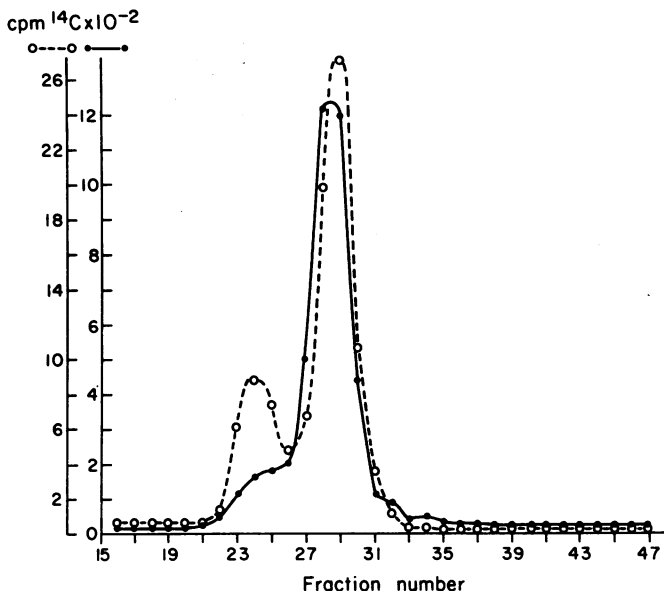

FIG. 4.—Possible structures accounting for the observed messenger activity.

(a) The "plus" strand, or free single-stranded RNA from f2 phage.

(b) The "plus" strand partially doubled up with a "minus" strand in the course of synthesis. Such structures probably arise as a first step in RNA replication.[3, 4, 27] They would be translatable preferentially for genes near the 5′-end of the "plus" strand.

(c) A "plus" strand in the process of synthesis, starting at its 5′-end.[3, 4] The resulting structure would be translatable preferentially for the gene nearest the 5′-end of the "plus" strand.

(d) A "plus" strand in the process of synthesis starting at its 3′-end.[27] This process would not require completion of the "minus" strand. If the latter were completed, the structure would be preferentially translatable for the gene nearest the 3′-end, whereas if the "minus" strand were

not completed, the gene nearest the 5′-end could be translated as well. It should be noted that the properties of these structures as regards translation are not affected by whether the new "plus" strand is synthesized by an asymmetric and semiconervative process or by a conservative one.

coat protein of f2 acts as a modulating factor *in vivo* by blocking the formation of noncoat phage proteins late in infection. There is some *in vitro* evidence in support of this model.[8, 9] We suggest that the structure of the multistranded intermediates of replication could also be responsible for the decreased synthesis of noncoat proteins observed *in vivo*. Since a proportion of the double-stranded RNA in infected cells has been found bound to polysomes,[6, 7] *in vivo* translation may occur from bound, partially double-stranded RNA. The double-stranded regions would specifically cover noncoat genes late in infection. Assuming that the coat gene is at the 5'-end, this would occur if progeny RNA is synthesized beginning at this 5'-end, leading to a structure with the coat gene selectively available (see Fig. 4c). However, other possible modes of synthesis will generate compatible structures (see Fig. 4d and Bishop *et al.*[27]). Completion of an intact progeny strand is here envisioned as followed immediately by its encapsulation into 'a phage particle. Thus most of the protein synthesis late in infection would normally occur upon partially double-stranded messengers. Coat protein would act, therefore, as a modulating factor by packaging the free RNA as soon as it is finished and preventing its further translation.

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