Translation of R-17 RNA Fragments

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Examination of the events during infection of cells by RNA phages reveals phemomena that are surprisingly complex for a virus that has only enough information to code for three to four proteins. The coat protein is synthesized at a rapid rate through most of the infectious cycle making it the predominant product. In contrast, the RNA synthetase is made at early times but not at late times (Viñuela et al., 1967a; Nathans et al., 1969). Since the RNA genome is itself messenger, the RNA phages present a unique opportunity to study translation of a polycistronic messenger. As first shown by Nathans et al. (1962) the major product of *f*2 directed in vitro protein synthesis is the coat protein, as is observed in vivo. Translation of the RNA synthetase cistron in vitro (Capecchi, 1966; Eggen et al., 1967; Viñuela et al., 1967b; Lodish, 1968) can be efficiently repressed by the addition of coat protein to the reaction mixture, (Eggen and Nathans, 1969; Sugiyama and Nakada, 1968; Lodish, 1968) suggesting that the coat protein acts as a translational repressor of the synthetase cistron. The third cistron, A (or maturation) protein, is not readily translated by the usual *E. coli* extracts. However, extracts from *B. stearothermophilus* seem to initiate synthesis of predominantly the A protein (Lodish and Robertson, this volume).

The existence of amber mutants in the coat protein cistron that are polar for the production of the RNA synthetase, both in vivo and in vitro, (Engelhardt et al., 1967; Capecchi, 1967), suggests that the availability of ribosome initiation sites is a factor in controlling differential translation of the cistrons in RNA phages. This is emphasized by the fact that only one ribosome can specifically bind to (Wahba et al., this volume) and initiate synthesis on (Webster et al., this volume) the native RNA. As has been pointed out (Zinder et al., 1966; Gussin et al., 1966), the polarity could be explained in two obvious ways. It could be the result of the obligatory movement of a ribosome from the 5′-end of the molecule to the 3′-end, which requires a specific gene order. Or, the three dimensional structure of the RNA could determine the availability of ribosome initiation sites, and this structure could be altered as a result of ribosome translocation, revealing new initiation sites. This involvement of three dimensional structure is strongly supported by experiments of Lodish and Robertson (this volume) showing independent translation of *f*2 cistrons upon modification of RNA structure.

We have recently shown that R-17 RNA can be cleaved by a relatively crude endonuclease preparation from *E. coli* into two specific fragments (Spahr and Gesteland, 1968): one carrying the 5′ terminus and 40% of the original RNA and the other representing the remaining 60% of the molecule. Here we describe some further characterization of these fragments and their messenger properties in vitro. This specific fragmentation of the R-17 RNA should allow the determination of the gene order by use of in vitro protein synthesis and ultimately by nucleotide sequence studies. We can also ask how the control of translation has been altered by fragmentation.

Preparation and Some Properties of the Endonuclease (RNase iv)

The endonuclease is isolated from the RNase I deficient strain *E. coli* MRE 600 (Cammack and Wade, 1965). It is important that the residual RNase I activity is at the lowest possible level which can be verified by testing the ribosomes for this activity (Gesteland, 1966).

Isolation procedure. Cells are grown at 37°C with vigorous aeration in a medium containing per liter: 10 g yeast extract, 34 g KH_2PO_4, 8.4 g KOH and 10 g glucose (the latter two sterilized separately). At an OD_{550} of about 10, the cells are still in log phase and are harvested, washed twice with 0.01 M Tris, pH 7.5 and 0.01 M magnesium acetate (TM buffer), and frozen in lots of about 100 g. A 20 liter culture yields about 200 g of cells (wet weight).

A crude extract is prepared by grinding with alumina (100 g of cells and 250 g of alumina in each of two large mortars) as described by Tissières

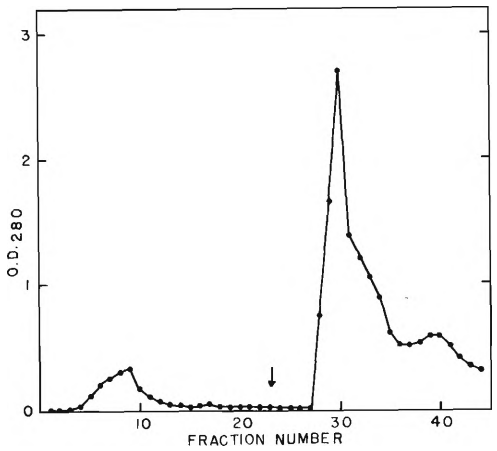


FIGURE 1. *DEAE cellulose chromatography of the endonuclease RNase IV.*

Column: 2.3 × 25 cm; fraction size: 20 ml; flow rate: 20–30 ml/hr; load: 1.28 g of protein from the ammonium sulfate step (see text). At the arrow the eluting buffer (0.01 M Tris, pH 7.5) was made 0.1 M in KCl. For other details see text.

et al. (1959). The extract is centrifuged immediately for 5 hr at 30,000 rpm in the Spinco 30 rotor and the entire supernatant fraction is collected. Its protein concentration is adjusted to 7.5 mg/ml by the addition of TM buffer.

To this solution, kept on ice, is added with stirring enough 10% (w/v) streptomycin sulfate to bring the final concentration to 0.48%. After 30 min of stirring, the suspension is centrifuged for 30 min at 9000 rpm in the large Sorvall rotor and the supernatant fraction is collected.

To this supernatant is added, while stirring on ice, enough 0.5% (w/v) protamine sulfate to reach a final concentration in protamine of 0.1%. After one hour the precipitate is centrifuged off (9000 rpm, 30 min) and the protein concentration of the supernatant is estimated. It should be less than 4 mg/ml and adjusted if necessary with TM buffer.

Ammonium sulfate (enzyme grade) is then added at 39 g/100 ml of the protamine supernatant. After one hour of stirring the precipitate is collected by centrifugation (9000 rpm, 30 min), dissolved in about 60 ml of 0.01 M Tris, pH 7.5, and dialyzed against the same buffer (two changes, 5 liters each, 5 hr). The protein concentration is estimated and the yield is usually 1.2–1.5 g of total protein.

This solution is applied to a DEAE cellulose column [Cellex D, Calbiochem, 0.6–0.7 meq/g, treated before use according to Peterson and Sober (1962)] buffered with 0.01 M Tris, pH 7.5, with dimensions of 2.3 × 25 cm. Because of the high protein concentration the sample has a tendency to produce channelling; to avoid this, the top 5 cm of the column is gently stirred with a glass rod after about 20 ml of the sample have been applied. Fractions of 20 ml are collected and the column is washed with about 500 ml of 0.01 M Tris, pH 7.5 at a flow rate of 20–30 ml/hr. The endonuclease, RNase IV, is then eluted with 0.01 M Tris, pH 7.5, containing 0.1 M KCl. The fractions containing the main part of the peak are pooled (fractions 28–31 of Fig. 1), and dialyzed against 0.001 M Tris, pH 7.5, (two changes of 5 liters for 5 hr each). The sample is lyophilized, dissolved in 10 ml of water, and clarified by centrifugation. The final yield is 80–100 mg. The preparation can be kept frozen at −18°C, or lyophilized and stored frozen. It is stable for at least one year.

The enzyme preparation is free of RNases I and II: when 1 mg is assayed on ^{14}C poly U (Spahr, 1964) there is no release of alcohol soluble material.

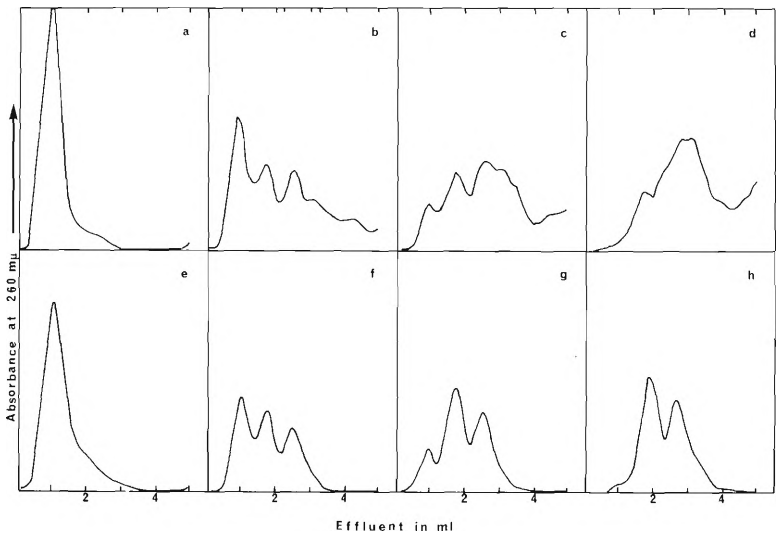


FIGURE 2. *Kinetics of R-17 RNA hydrolysis by RNases I and IV.*

RNase I was purified from ribosomes of *E. coli* B following the procedure described by Spahr and Hollingworth (1961) with a slight modification of the last step (chromatography on amberlite): no albumin was added to the collection tubes and as soon as the enzyme was eluted the active fractions were pooled, precipitated with ammonium sulfate (0.9 saturation) and centrifuged for 3 hr at 30,000 rpm after standing for 2 hr in the cold. The barely visible precipitate was dissolved in 0.5 ml of 0.01 M Tris.

Enzymatic degradation of R-17 RNA and the sucrose gradient analysis have been described (Spahr and Gesteland, 1968).

a–d: RNase I, 0.01 units *a*: no enzyme 10 min, *b*: 1 min, *c*: 3 min, *d*: 6 min.

e–h: RNase IV, 10 μl *e*: no enzyme, 60 min, *f*: 30 min, *g*: 60 min, *h*: 80 min.

No polynucleotide phosphorylase activity could be detected when 2 mg of the preparation is assayed (Spahr, 1964, procedure A).

The endonuclease RNase IV is active in the pH range 5–9 with an optimum between 6 and 8 when assayed under the standard conditions (see legend of Fig. 2). The molecular weight of the enzyme estimated from gel filtration is 31,000.

Mode of Action

RNase IV hydrolyzes RNA very slowly to large oligonucleotides but does not release acid soluble nucleotides. It appears to be inactive on DNA: incubation of 1 mg of enzyme with 2 μg of tritium labeled T2 DNA (20,000 dpm) for one hour at 30°C causes no change in the sedimentation pattern of the DNA on sucrose gradients. The endonuclease is inactive on poly A, poly U, and poly C, when incubated under the standard conditions for one hour with an enzyme/substrate ratio of 10. *E. coli* 16 S and 23 S ribosomal RNA are hydrolyzed slowly and randomly.

Of particular interest is the action of RNase IV on R-17, MS-2, and *f*2 RNAs, which are initially cleaved into two fragments representing about 60% and 40% of the original molecule. In the case of R-17 RNA, which has been most thoroughly studied, the point of cleavage is about 1400 nucleotides in from the 5′-end of the molecule (see below). With MS-2 RNA it is known that the smaller fragment carries the original 5′-end of the RNA and that the larger has the original 3′-terminus, but the molecular weights have not been determined (Min Jou et al., 1969). The fragments produced from *f*2 RNA have the same sedimentation coefficients as those from R-17, but they have not been further characterized.

Pancreatic RNase in trace amounts will cleave Qβ RNA (Bassel and Spiegelman, 1967) and R-17 RNA (Thach and Boedtker, pers. commun.) initially producing some fragments that sediment like those obtained with RNase IV, but this action differs at least in the case of R-17 RNA in that other points of the molecule are attacked almost simultaneously. A similar mode of action is observed when *E. coli* RNase I is incubated with R-17 RNA. Figure 2 compares the kinetics of R-17 RNA hydrolysis by RNase I and RNase IV. In the initial stage of degradation the sedimentation profiles show two new peaks in both cases but the relative proportions are different (about equal in the case of RNase I), and RNase I produces slower sedimenting material. The differences are more marked at later times. Thus in Fig. 2*d* and *h*, when no RNA remains intact, RNase IV has produced two specific peaks, whereas the degradation by RNase I is practically random. Thus, there appears to be a region of the RNA phage RNA molecules that is particularly susceptible to ribonucleases, and in the case of RNase IV there seems to be a great preference for splitting at this point, since as much as 75% of the RNA can be degraded to yield predominantly the two fragments (Fig. 2*g*).

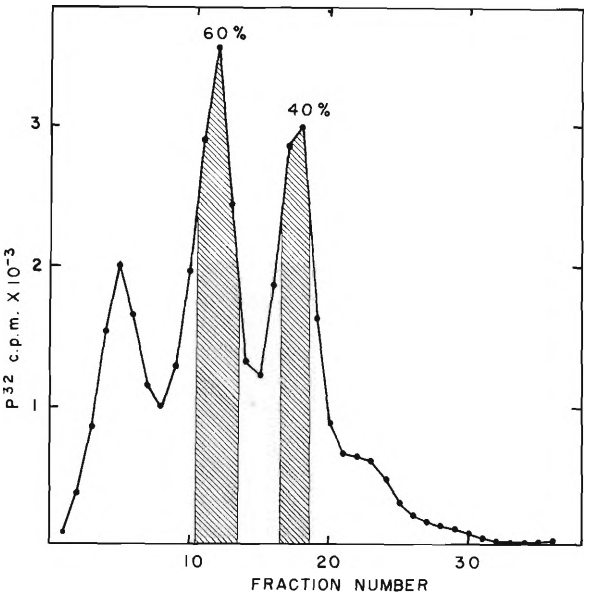


FIGURE 3. *Sucrose gradient purification of R-17 fragments.* Preparation of the ^{32}P labeled R-17 RNA and degradation with RNase IV have been described previously (Spahr and Gesteland, 1968). The 1 ml reaction mixture contained 100 μmoles sodium citrate, pH 6.0; 5 μmoles ethylenediaminetetraacetic acid; 1 mg ^{32}P R-17 RNA (13×10^6 count/min) and 0.16 ml RNase IV as described in the text. After incubation for 25 min at 30°C, sodium dodecylsulfate and phenol were added to extract the RNA which was then precipitated once with alcohol, dissolved in 0.1 M NaCl, 0.01 M sodium acetate, pH 5, and layered on a 38 ml (Spinco SW 27 rotor), 5–20% sucrose gradient made up in the same buffer. Fractions were collected after centrifugation for 19 hr at 27,000 rpm and 4°C. Aliquots of 1 μl were diluted into water and counted directly in a scintillation counter. Peak tubes were pooled as shown, and the RNA was precipitated with 2.5 volumes of ethanol by storage overnight at −20°C. The precipitate was collected by centrifugation at 19,000 rpm in a Sorvall centrifuge. After draining the tubes for one hour the precipitate was dissolved in 1 mM sodium acetate, pH 5, and frozen.

Specific Fragments of R-17 RNA

Preparative sucrose gradient centrifugation gives sufficient resolution to permit isolation of relatively pure RNA fragments; as shown previously (Spahr and Gesteland, 1968; Spahr, Farber, and Gesteland, 1969), and as shown in Fig. 3 (this is aided by use of the SW27 Spinco rotor). The RNA from the pooled sucrose gradient fractions, after ethanol precipitation and dissolution, can be stored frozen and repeatedly thawed and refrozen without apparent

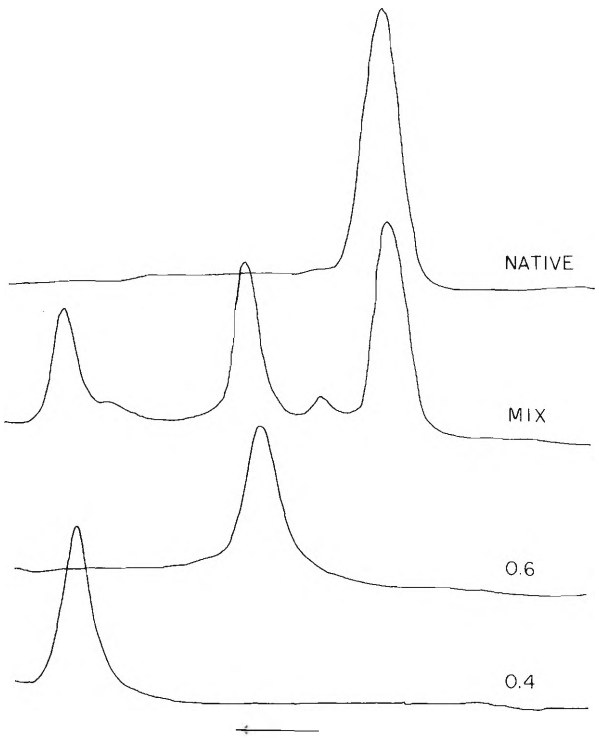


FIGURE 4. *Gel electrophoresis of RNase IV products.*
Unlabeled R-17 RNA was degraded as described in Fig. 3. An aliquot of the reaction mixture was analyzed electrophoretically on a 2.6% polyacrylamide gel as described previously (Spahr, Farber, and Gesteland, 1969), in parallel with samples of the native RNA and the purified preparations of the 60% and 40% fragments (see Fig. 3). The migration is towards the anode on the left.

harm. Figure 4 shows acrylamide gel electrophoresis of the original degradation products compared with the purified fragments. In addition to the major 60% and 40% fragments, the initial mixture contains two minor components. The amounts of these species are variable and they are undetectable with some enzyme preparations. Purification of the fragments gets rid of the majority of these contaminants as shown in Fig. 4.

Our early results (Spahr and Gesteland, 1968) showed that the 5′-pppGp was retained in the 40% fragment and that the 60% fragment was deficient in pppGp, but retained enough of it so that perhaps 20% of the molecules could have had the 5′-end of the native RNA. What is the origin of this pppGp? Is it due to contamination of the 60% fragment with native or 40% RNA, or is it due to enzymatic cleavage at a second point that produces some RNA fragments, 60% in length, but from the 5′-end of the molecule? As will be seen, this becomes critical when trying to interpret the results of in vitro protein synthesis with the fragments. We have looked more carefully at this point, using ^{32}P labeled fragments prepared from only peak fractions of the sucrose gradient, and subjecting these to gel electrophoresis and pppGp analysis. The electrophoresis results shown in Fig. 5, indicate that the 40% fragment is virtually free of contamination with larger RNA molecules. The 60% fragment is about 80% homogeneous, having no detectable native RNA and about 7–10% RNA that is the size of the 40% fragment, plus some intermediate sized pieces. Table 1 gives the results of pppGp analyses on these same fragment preparations. From molecular weights of the fragments, determined by gel electrophoresis and sedimentation velocity in formaldehyde (Spahr, Farber, and Gesteland, 1969), one can calculate that the 60% fragment has 2100

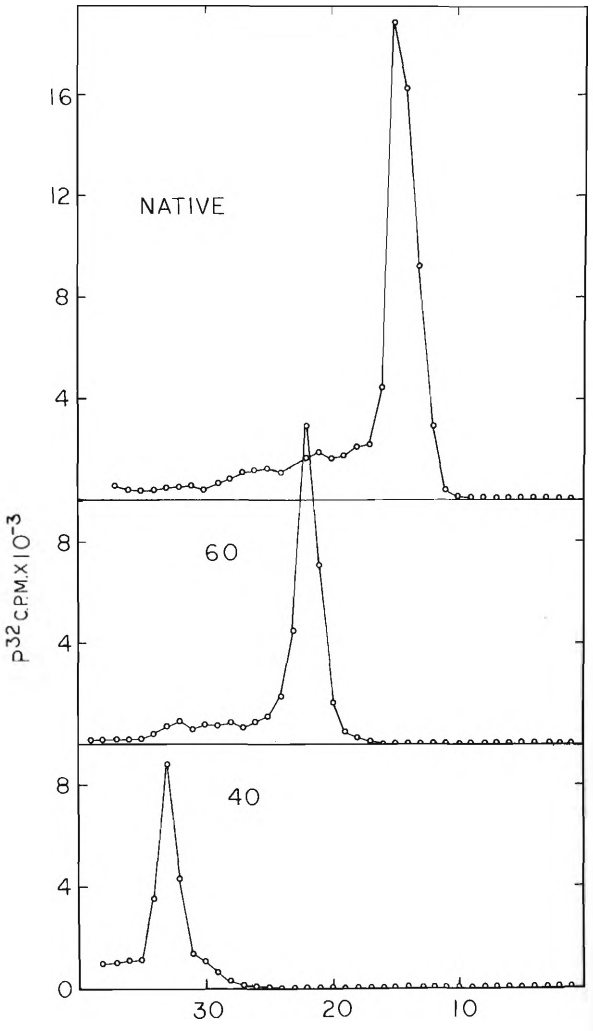


FIGURE 5. *Gel electrophoresis of ^{32}P fragments.*
Aliquots of the ^{32}P labeled 60% and 40% fragments from the sucrose gradient shown in Fig. 3 were subjected to electrophoresis as in Fig. 4. The gels were frozen and sliced into 1.5 mm sections using a modified microtome with a freezing stage. The radioactivity was determined by counting the slices directly in water using a liquid scintillation counter.

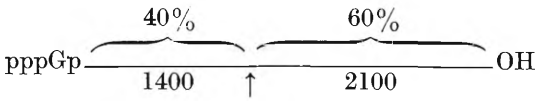
TABLE 1. ANALYSES OF pppGp OF ^{32}P RNA

	Native RNA	60%	40%
Number of nucleotides	3500	2100	1400
Radioactivity in pppGp (%)	0.081	0.021	0.210
Chains with pppGp (%)	71	(11)	73

The analysis of pppGp was carried out on the same fragment preparations shown in Fig. 3 as described by Roblin (1968) using DEAE Sephadex chromatography in 8 M urea. The percentages of chains carrying pppGp are calculated using the indicated number of nucleotides per RNA molecule. Thus, if in the 60% fragment 0.021% of the radioactivity were in pppGp, then only 11% of the chains would bear this group.

nucleotides and the 40%, 1400 compared to 3500 for the native RNA. Using these numbers, the data of Table 1 show that 71% of the chains of the native RNA carried pppGp, as has been found previously (Roblin, 1968; Spahr and Gesteland, 1968), and that 73% of the 40% chains have pppGp, showing that these come exclusively from the 5′-end of the molecule.

The 0.021% of the pppGp found in the 60% fragment preparation could mean that 11% of the 2100 nucleotide chains have pppGp termini. On the other hand the gel electrophoresis (Fig. 5) shows that this 60% preparation has 7–10% contamination with material the size of the 40% piece, which would contribute about this amount of pppGp if it were like the standard 40% preparation. Thus, it seems most likely that the pppGp in the 60% fragment preparations can be accounted for to some extent by contamination with 40% fragment. Since the 40% fragment has the full predicted complement of pppGp, we conclude that the only detectable cleavage under these conditions is:



This does not mean to imply that the enzyme attacks only one particular site producing unique new ends, but only that the cut occurs within this region producing fragments with *average* size of 1400 and 2100.

IN VITRO TRANSLATION

Both R-17 RNA fragments are able to stimulate protein synthesis in vitro however, with a lower efficiency than the native RNA. While the 60% fragment retains about 60% of the specific messenger activity, the 40% fragment is only 10–15% as active as the whole RNA. This holds true both for total amino acid incorporation (Spahr and Gesteland, 1968) and for initiation as measured by incorporation of .(^{35}S)N-formyl-methionine [(^{35}S)F-met] into peptides (see below). The inefficiency of the 40% fragment is difficult to understand, since it has the normal 5′-end of the RNA and since it is efficient at specifically binding to ribosomes (Miller and Wahba, pers. commun.).

Upon examination of the products of in vitro protein synthesis on polyacrylamide gels, it was readily seen that the 60% fragment stimulated synthesis of the complete RNA synthetase protein (Spahr and Gesteland, 1968). That the protein peak seen on the gels in the position of synthetase is indeed this protein is shown by the fact that RNA from an amber mutant of R-17, C-13, known to be in the synthetase cistron (Gussin, 1966), fails to make this protein in vitro, and that addition of suppressor tRNA causes a slight reappearance of the peak (data not shown).

The gel patterns also suggested that both pieces were able to stimulate synthesis of some protein that, like the coat protein, lacked histidine and moved rapidly on electrophoresis. The amount of this material however, was small compared to the large amount of coat synthesized by native RNA. Identifiable A protein was not found in the products even with native RNA.

In order to have a more sensitive and specific assay for the proteins made in vitro, we have employed the system described by Lodish (1968, and Lodish and Robertson, this volume) of labeling the amino termini of the in vitro synthesized product by using (^{35}S)F-met transfer RNA (tRNA). We have taken the acid insoluble material from protein synthesizing reaction mixtures, oxidized this with performic acid to avoid confusion about methionine derivatives, and digested the mixture with trypsin and chymotrypsin (see legend to Fig. 6). The peptides are separated on cellulose thin layer plates using electrophoresis at pH 3.5 in the first dimension and chromatography in the second. The peptide pattern observed using native R-17 RNA as template is similar to that found for *f*2 by Lodish (1968), but simplified by oxidation and apparent lack of deformylated derivatives. The two major peptides (90–95% of the radioactivity) are the initiating sequences for the coat protein and the RNA synthetase as shown by the following facts. 1. If the peptides are eluted and treated with pronase, most of the radioactivity moves in electrophoresis and chromatography with authentic F-met-Ala in one case and F-met-Ser in the other. 2. The predominant peptide is F-met-Ala . . . (Table 2) which is known to be the initial sequence of in vitro made coat protein (Adams and Capecchi, 1966; Webster et al., 1966). 3. The synthesis of the F-met-Ser . . . peptide is repressed by the addition of coat protein to the reaction mixture (Fig. 6 and

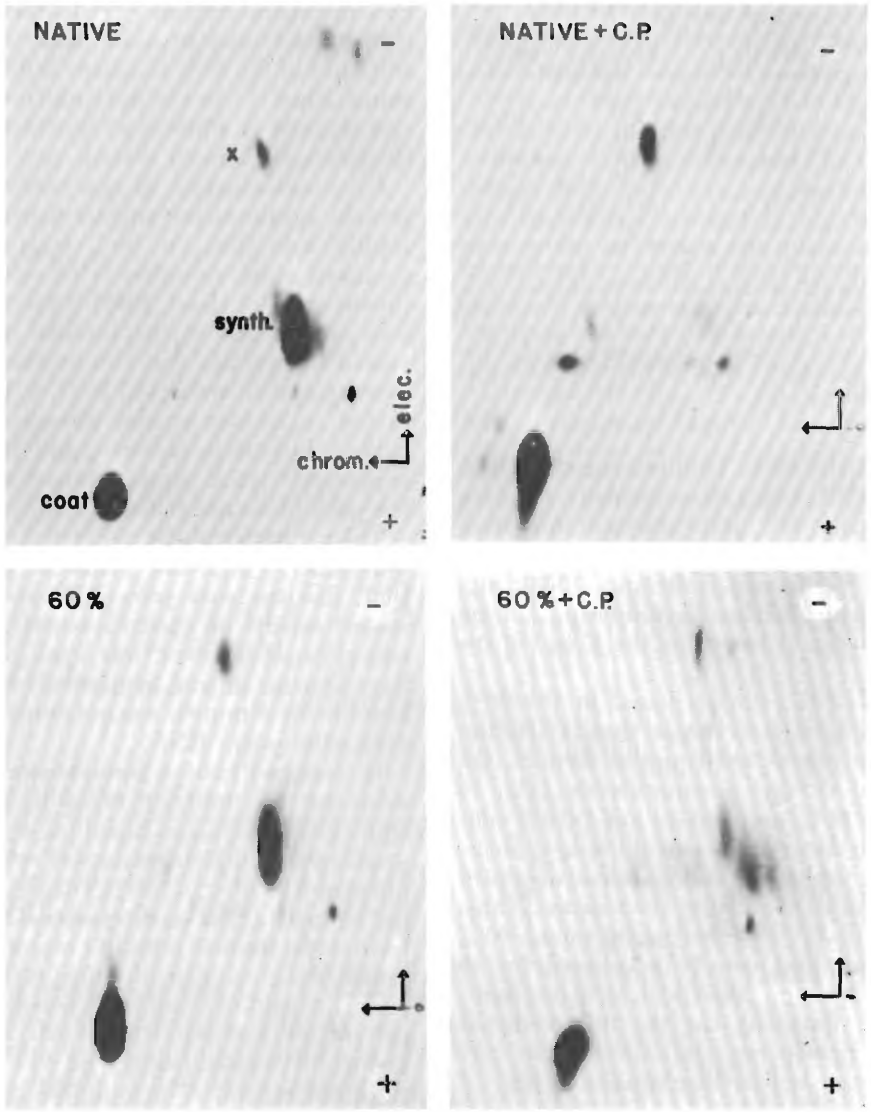


FIGURE 6. *Fingerprints of* (^{35}S) *methionine peptides synthesized in vitro.*

Protein synthesis in vitro was carried out as described previously (Salser, Gesteland, and Bolle, 1967), but using F-(^{35}S)met-tRNA as a source of label and treating the samples as described by Lodish (1968). The synthesis was stimulated by the addition of native R-17 RNA, 60% fragment, or 40% fragment (see Table 2) each at a final concentration of 150 μg/ml. R-17 coat protein was prepared from purified phage as described by Sugiyama et al. (1967), and when used it was added to the reaction mixtures before the S-30 extract (see below). Twenty molecules of coat protein were added per molecule of messenger.

The F-(^{35}S)met-tRNA was made according to Marcker (1965) using tRNA purified (from RNase I_{10}^-) as described by Capecchi (1966). High specific activity (^{35}S)methionine (about 100 c/mmole) was isolated from *E. coli* grown on sulfur deficient medium in the presence of radioactive sulfate (to be published).

The reaction mixture for protein synthesis (final volume of 0.1 ml) contained per milliliter: 250 μl of pre-incubated S-30 extract from *E. coli* RNase I_{10}^-; 62.5 μmoles Tris, pH 7.8; 55 μmoles NH_4Cl; 2.25 μmoles dithiothreitol; 9.0 μmoles magnesium acetate; 0.13 μmoles of each of 20 amino acids; 5 μmoles phosphoenolpyruvate; 3 μmoles ATP; 0.2 μmoles GTP; 20 μg pyruvate kinase; 5 μmoles polyethyleneglycol, 6000; 100 μg tRNA containing the charged species F-(^{35}S)-met-tRNA (6.5×10^6 count/min). After incubation for 12 min at 36°C, each 0.1 ml reaction mixture received 13 μg pancreatic RNase and 1 μmole EDTA and was incubated for 10 min at 36°C and precipitated with 5% TCA. After two TCA washes, two ethanol:ether (1:1) washes and two ether washes, the samples were lyophilized, oxidized with performic acid (Capecchi, 1966), lyophilized, digested with trypsin and chymotrypsin (Lodish, 1968), lyophilized, and finally dissolved in 25 μl of electrophoresis buffer (pyridine:acetic acid:water, 1:10:189, pH 3.5). Finger printing was done on microcrystalline cellulose thin layer plates (20×20 cm). A 10 μl sample was spotted in a 2 cm strip and electrophoresis was carried out in the first dimension, using the above buffer, on a cooling plate at 20°C for 100 min with 600 v. The 2 cm bands were sharpened in the second dimension using 1% acetic acid, and ascending chromatography was carried out using iso-amyl alcohol:pyridine:water (35:35:30), (Lodish, 1968). The plates were dried and radioautographed on Kodak X-ray film for 4–5 days.

TABLE 2. RADIOACTIVITY IN INITIATION PEPTIDES

Messenger	Coat peptide (F-met-Ala . . .)	Synthetase peptide (F-met-Ser . . .)
Native RNA	7210	2550
Native RNA + coat protein	8085	374
60% fragment	2002	1935
60% fragment + coat protein	2296	231
40% fragment	220	420
40% fragment + coat protein	161	290
No RNA	31	780
No RNA + coat protein	68	662

The radioactive peptides of Fig. 6 were scraped from the thin layer plates and counted in toluene scintillator. The peptides are identified in Fig. 6 (upper left) and the corresponding areas were counted from the other plates.

Table 2) as is seen in the case of *f*2 (Lodish, 1968). 4. If the in vitro made product is fractionated on polyacrylamide gels before oxidation and fingerprinting, the F-met-Ala. . . containing material is found only in the region of authentic coat protein and the majority of the F-met-Ser . . . containing material migrates more slowly to the expected position for the RNA synthetase. We have not identified the A protein initiation peptide although there are a number of minor components (see below).

With no RNA added to the in vitro system one observes some radioactivity in the region of the synthetase initiation peptide (Fig. 6 and Table 2). With native RNA as messenger in the presence of excess coat protein, the synthetase peptide is reduced to a level below that of the control sample without RNA. The coat protein peptide is unaffected by the presence of the coat protein. The 60% fragment stimulates initiation of synthetase about as well as the native RNA, and this too is ininhibited by the addition of coat protein to the reaction mixture. In addition, the 60% fragment stimulates coat initiation, but at about 1/4 the efficiency of the native RNA. The 40% fragment, by contrast, initiates little if any coat and no synthetase compared to the sample without RNA.

Loss of Polarity

An R-17 amber mutant in the coat protein cistron ($am\mathrm{B}_2$) at position six exerts a polar effect on the synthetase cistron (Gussin, 1966). This effect can be demonstrated in vitro (Capecchi, 1967) by measuring the incorporation of histidine (not present in the coat) into phage proteins as a function of magnesium ion concentration. At low magnesium levels in a nonsuppressing extract, the polar mutant RNA stimulates the incorporation of only one-tenth to one-seventh as much histidine as the wild type RNA. This difference is practically eliminated by use of suppressing extracts or at high magnesium levels (in a nonsuppressing extract) where the fidelity of translation is impaired.

If such a polar effect could be observed with either of the fragments it would suggest the location of the coat cistron. Figure 7 gives the results of such an experiment using fragments from $am\mathrm{B}_2$. As observed by Capecchi (1967), in the su^- system at low magnesium, the polar amber mutant RNA directs only about one-eighth as much histidine as the wild type RNA (Fig. 7*a*, 8 mM magnesium), and this is eliminated by suppression (Fig. 7*b*). However, neither of the two fragments derived from $am\mathrm{B}_2$ RNA exhibit this polarity of histidine incorporation (Fig. 7*c–f*). Apparently fragmentation results in loss of polarity.

DISCUSSION

The initiation assay demonstrates that the 60% fragment preparations do have the amino terminal information for the coat protein. However, since the level of this initiation is reduced compared to the native RNA, there are several possible trivial explanations that must be entertained. First, this could be due to contamination of the 60% fragment with the native RNA to the extent of 25%. This is ruled out by the gel electrophoresis results of the purified fragments shown in Fig. 4 and 5, where such contamination is not observed. Second, contamination with smaller RNA pieces that carry this information could be responsible. Some lower molecular weight species are clearly seen in the 60% preparations, but since there is a general loss of the ability to initiate coat upon fragmentation, this seems unlikely. Third, the coat initiation could be from 60% fragments of a different origin from the majority of the fragments, for instance 60% pieces from the 5′-end of the RNA. As discussed above, the pppGp analyses are consistent with the inference that about 11% of the 60% fragments have pppGp and thus possibly come from the 5′-end. However, contamination with 40% fragment as seen on the gels could account for this amount of pppGp. Therefore there is no evidence for cleavage at a second site to produce 5′ 60% pieces, so this explanation for coat protein initiation by 60% fragments seems unlikely. If these possible explanations can be ruled out, then we must conclude that the 60% piece from the 3′-end does contain the coat initiation site.

Why then is the level of coat initiation with this fragment lower than with the native RNA? The RNase IV could act at more than one site within a rather narrow region producing on the average 60% and 40% fragments, but cutting on either side of

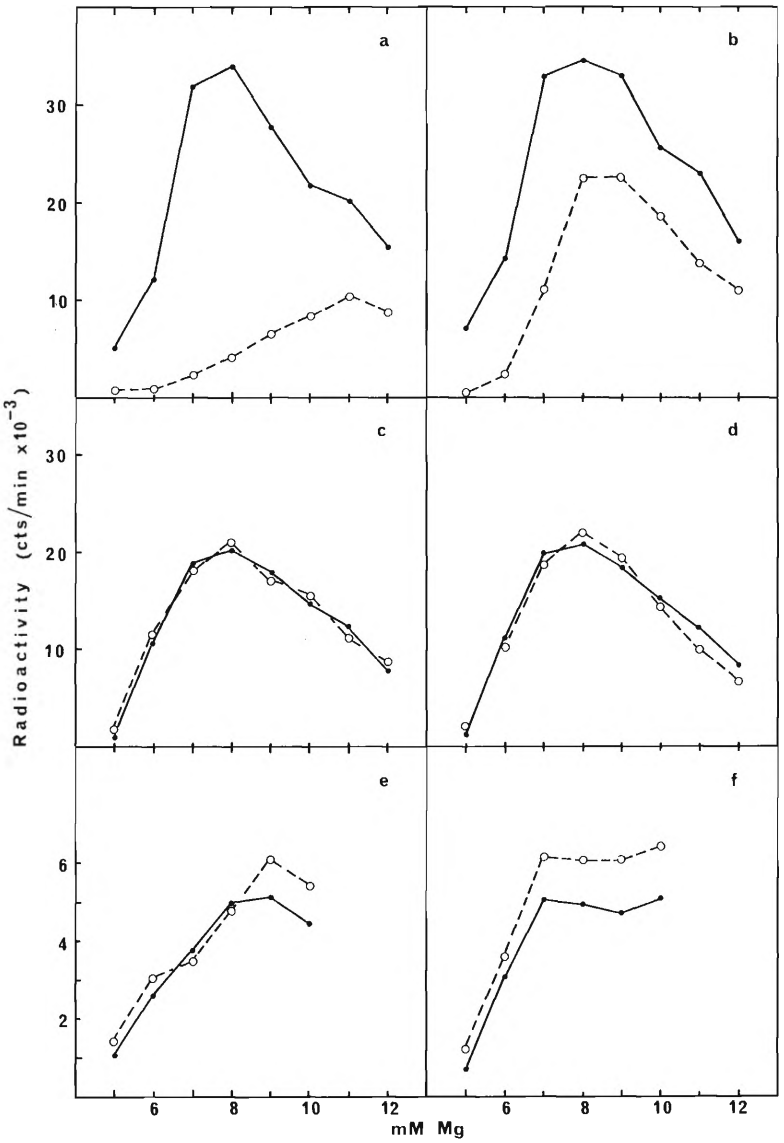


FIGURE 7. *Polarity in vitro with* amB_2 *R-17 RNA.*

Magnesium ion dependence of (^{3}H) histidine incorporation into polypeptide stimulated by wild type R-17 RNA and its fragments (–●–●–●–) and by the polar mutant *am*B_2 RNA and its fragments (–○ – – ○ – – ○ –) using *su*$^-$ and *su*$^+$ cell free extracts.

a: Native RNAs in *su*$^-$ extract. *b*: Native RNAs in *su*$^+$ extract. *c*: 60% fragments in *su*$^-$ extract. *d*: 60% fragments in *su*$^+$ extract. *e*: 40% fragments in *su*$^-$ extract. *f*: 40% fragments in *su*$^+$.

The reaction mixtures were as described in Fig. 6, with the additions of preincubated S-30 extracts from *E. coli* S-26 (*su*$^-$); from 5–12 μmoles magnesium acetate/ml; 0.14 μmoles of each of 19 amino acids (minus histidine); 1.1 μc (^{3}H) histidine (4 mc/μmole); and 6 μg tetrahydrofolic acid. In the experiments *b*, *d* and *f* this *su*$^-$ cell free system was supplemented with *su*$^+$ tRNA from the suppressor strain S26R1E, as described by Capecchi (1966), to a final concentration of 80 μg per 0.1 ml reaction mixture. Wild type or *am*B_2 RNA (32 μg), 60% fragments from wild type or *am*B_2 (35 μg) and 40% fragments from wild type or *am*B_2 (35 μg) were added to the 0.1 ml reaction mixtures. After incubation for 15 min at 36°C, the reaction was stopped by the addition of 0.1 ml of 1 N KOH and a further incubation was carried out for 15 min to hydrolyze the aminoacyl tRNA. The TCA precipitable material was collected on glass fiber filters and counted in a scintillation counter.

the coat initiation region. If this is so, then the 40% fragment should often include the coat site, but we have not detected this in our experiments. Possibly the coat protein fragment is too short to be precipitated by acid and consequently has been missed. Or, possibly the short coat region near the 3′-end of the 40% fragment cannot be translated efficiently if, for instance, this end is rapidly degraded by exonuclease activity such as RNase II in the in vitro system.

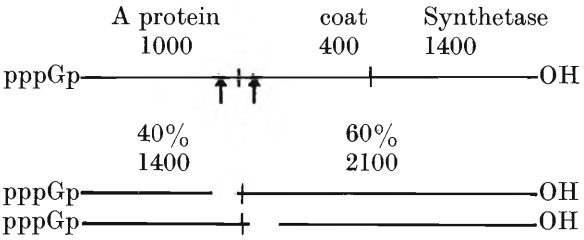
Alternatively, it could be that the RNase IV always cuts in the same place and leaves the coat site on the 60% piece, and the altered initiation ratio is due to changes in the availability of the ribosome binding sites. Structural changes accompanying fragmentation could open up the RNA, which would make the sites more available and result in equal and independent initiation frequencies, as Lodish has observed with formaldehyde-treated *f*2 RNA (Lodish and Robertson, this volume).

We cannot distinguish between the two alternative explanations for the decreased coat protein initiation by the 60% fragment. The lack of polarity of the coat protein amber mutant fragments is consistent with both possibilities. If the 60% fragment does not always have the complete coat gene then those molecules lacking part of it would show independent translation of the histidine containing synthetase and hence no polarity. On the other hand, if the coat and synthetase have equal initiation rates caused by changes in secondary or tertiary structure, then these changes might also be responsible for the loss of polarity in

keeping with the model for polarity (Gussin, 1966; Zinder et al., 1966; Lodish and Robertson, this volume) in which the structure of the RNA prevents initiation at the synthetase cistron until the coat cistron is read.

Independent of the location of the coat protein cistron on the RNA, the initiation site for A protein must be in the 40% fragment. The synthetase takes up about 1400 nucleotides of the 2100 in the 60% fragment, not leaving enough information for the complete A protein (1000 nucleotides) so its initiation region must be within the 40% fragment, although it has not been detected in these experiments (however, see Steitz, this volume).

The picture that is most consistent with our data is:



The experiments that suggest this however are indirect, since they use in vitro protein synthesis. There is always uncertainty about the fraction of RNA molecules that actually participate in protein synthesis and about the effects of the substantial nuclease activity in the extracts. Therefore, this order is only tentative and must await quantitative experiments.

Information about the gene order has come from many sources, and if one makes the only reasonable assumption that the closely related RNA phages all have the same gene order, then the data are conflicting. Shimura, Kaizer, and Nathans (1968) have used incomplete RNA molecules of phage MS-2 grown in fluorouracil, which lack about one-third of the 3′-end of the RNA, to direct in vitro protein synthesis. They find that the fragment no longer makes the RNA synthetase, which places this cistron nearer the 3′-end than the coat protein, and agrees with the interpretation above.

On the other hand, a number of experiments with *f*2 RNA strongly imply that the coat cistron is nearest the 5′-end. Lodish (1968) has shown that a 55–70% fragment, isolated from defective phage particles, comes from the 3′-end of the molecule. He finds that this RNA initiates maturation (or A) protein and synthetase, but not coat protein, as if the coat gene were nearest the 5′-end. Consistent with this is the experiment of Webster et al. (this volume) that shows that the single ribosome bound in the first part of the coat cistron protects a 120 nucleotide fragment containing the pppGp from the action of venom phosphodiesterase. This implies that the coat initiation site is in the first 120 nucleotides. The nucleotide sequence results of Adams et al. (this volume) show that the coat protein initiation sequence is not in the first 100 nucleotides of the 5′-end. In fact, Billeter et al. (this volume) find no such site within the first 160 nucleotides of the 5′-end of Qβ RNA, which is consistent with the finding of Bassel (1968) that a 2/3 fragment from the 3′-end of Qβ still makes coat protein in vitro.

It seems clear that only more data concerning nucleotide sequence will unequivocally establish the gene order. Experiments are in progress in collaboration with J. Argetsinger Steitz and P. Jeppeson, using the fragments for sequence studies. Their preliminary experiments (see Argetsinger Steitz, this volume) show that the 40% fragment has the A protein initiation site and considerable coat initiation site while the 60% piece has the synthetase initiation site. However, the 60% fragment appears to have the T_1 oligonucleotide coding for amino acids 89–95 of the coat protein as identified by Adams et al. (this volume). If this proves to be correct it would provide the best support for the gene order proposed above.

The evidence of Webster et al., (this volume) strongly says that only one ribosome binds initially to the native RNA and this is at the coat protein site. The other sites are apparently inaccessible, but can be revealed by unfolding the RNA (Lodish and Robertson, this volume) or by fragmentation. Sequence data (Billeter et al.; Adams et al.; Steitz; this volume) show that secondary structure loops are very prevalent, which suggests that these, plus tertiary folding, may govern the accessibility of sites not only for ribosome initiation but also for nuclease attack. If so, then according to the model proposed above, it should not be surprising that RNase IV (and other nucleases) would tend to cleave the RNA at the exposed coat protein initiation region. Sequence studies are clearly needed to characterize the newly created ends of the fragments.

The structure of the 60% fragment is altered upon cleavage of the native RNA in that the availability of ribosome sites is changed. However, the 60% fragment still binds one molecule of coat protein per molecule of RNA (Spahr, Farber, and Gesteland, 1969) and translation of the synthetase cistron in this fragment is still completely repressed by excess coat protein. So this aspect of the RNA structure is retained during fragmentation. The results above, like those of Lodish (1968), show that the coat protein repression acts at a very early stage of translation of the synthetase cistron, as it prevents formation of the initiation peptide.

Perhaps the coat prevents ribosome binding at this site, but it is clear that although the coat binds perfectly well to the native RNA (Sugiyama et al., 1967; Spahr, Farber, and Gesteland, 1969; Eggen and Nathans, 1969) ribosomes will not initiate at the synthetase site even in the absence of coat protein.

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