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Differential Synthesis of Bacteriophage Proteins Directed by a Fragment of MS2 RNA

Yoshiro SHIMURA

The Research Institute for Microbial Diseases

Osaka University, Osaka, Japan

ABSTRACT The RNA fragment isolated from defective, 5-fluorouracil (FU) containing MS2 phage particles consists of about two-thirds the normal length and appears to come from the 5′ end of the phage RNA. This fragment codes for the phage coat protein and probably the maturation protein. It does not direct the synthesis of a protein corresponding to the RNA synthetase. This result suggests that the synthetase cistron is at or near the 3′ end of the phage RNA molecule.

Introduction

MS2 is one of the small RNA coliphages, first isolated by Loeb and Zinder[1], and has single-stranded RNA of 1.1×10^6 molecular weight[2,3]. Therefore the number of proteins encoded by the viral RNA is small; in fact three viral proteins have been detected in infected cells, corresponding to the three known complementation groups.[4,5] These proteins have also been detected among the protein products made in a Nirenberg-type *E. coli* extract programmed with MS2 RNA as messenger.[4,6] It thus became possible to test *fragments* of phage RNA for their ability to direct the synthesis of specific phage proteins. By this means it is hoped to determine the position of specific cistrons.

Fragments of phage RNA have been obtained by the following three methods:

1. From RNA-deficient particles which are produced by adding 5-fluorouracil (FU) to infected culture.
2. By limited digestion of phage RNA with ribonuclease T1.
3. From defective amber mutants which are deficient in RNA, as shown by Lodish et al..[7] by Heisenberg[8] and by Argetsinger and Gussin.[9]

This paper describes the properties of RNA fragments which have been examined in more detail; namely the fragment isolated from defective particles produced in the presence of 5FU. The results to be presented indicate that the RNA-synthetase cistron is at or near the 3' end of the phage RNA molecules.

A summary of part of this work has already appeared.[10]

Materials and Methods

MS2 and its host, *E. coli* C3000, were obtained from R. L. Sinsheimer. The procedures for growth, purification and assay of phage were those described previously.[11]

Protein synthesis in *E. coli* extracts and analysis of the products were as described previously[4,12] except that electrophoresis of tryptic peptides was done at pH 3.6 in pyridine acetate (pyridine-acetic acid-water, 1:10:280) and that *E. coli* Q13 (kindly provided by W. Gilbert) was used to prepare cell extracts. The protein products were solubilized by treating the incubation mixture with sodium dodecyl sulfate (SDS: final concentration 1%), ethylenediaminetetraacetate (0.05 *M*) and mercaptoethanol (0.05 *M*). After 1 hour at 37°C, 1/20 volume of 0.1 *M*-C^{12}-amino acids was added, and the samples were dialyzed on a rapid, semi-micro-dialyzer against 0.1% SDS-0.01 *M*-mercaptoethanol. At the end of dialysis, 1/4 volume of 60% sucrose and 1/100 volume of 1 *M*-potassium phosphate, pH 7.2, were added and the samples electrophoresed in 10% acrylamide gels.[6]

Radioactive proteins in the gel were detected by two procedures : radioautography of dried slices of gel, as described by Fairbanks, Levinthal, and Reeder[13] or by slicing the gel into 1-mm segments, eluting with 0.1% SDS, and counting the eluted protein in a liquid scintillation counter. The radioautograms were traced with a Joyce-Loebl recording microdensitometer.

Determination of protein was by the phenol method[14] using bovine serum albumin as a standard. Total RNA was determined by the orcinol method[15] and base composition, by optical density of the 2',3'-nucleotides following hydrolysis of the RNA with KOH and separation of the nucleotides by paper electrophoresis at pH 3.5.[16]

Percentage replacement of uracil by FU was determined on alkaline hydrolysates of (^{32}P)-labelled FU-RNA by counting $UM^{32}P$ and $FUM^{32}P$ separated by electrophoresis on Whatman 3MM paper at 35 v/cm for 2hr in borate buffer at pH 9.3.[17]

Determination of nucleoside tetraphosphate involved the preparation of ^{32}P-labelled RNA purified on sucrose gradient, alkaline hydrolysis and electrophoresis on Whatman 3MM paper in 0.05 *M*-sodium citrate at pH 5.4 as described by Bremer et al.[18]

Source of chemicals. 5-Fluorouracil was kindly supplied by Dr. R. Duschinsky of Hoffman-La Roche, Inc., Nutley, N. J., U.S.A. Uniformly labelled (^{14}C)-amino acids were obtained from the New England Nuclear Corp., Boston, Mass., and tritiated amino acids were from the Nuclear Chicago Corp., Chicago, Ill. or from Schwarz BioResearch, Inc., Orangeburg, N. Y., U.S.A.

Results

(a) Formation of defective particles

When FU is added, after a critical time, to a culture infected with MS2

phage, phage particles containing FU are produced.[11,19] As seen in Fig. 1, there are two classes of such particles which have different buoyant density: i.e. heavy phage particles and particles of low buoyant density. At the concentration of FU used in these experiments, 80% of the uracil is replaced by FU.[11] These

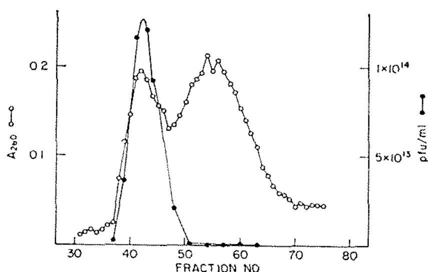


Fig. 1 CsCl gradient equilibrium sedimentation of FU particles. *E. coli* C 3000 was infected with MS2 as previously described[11] and 30 μg/ml FU added 17 minutes after infection. The progeny phage was purified as described and the CsCl fractions analysed for infective phage (●) and A 260 (○). Normal MS2 gave a single optical density peak with slight tailing on the light side.

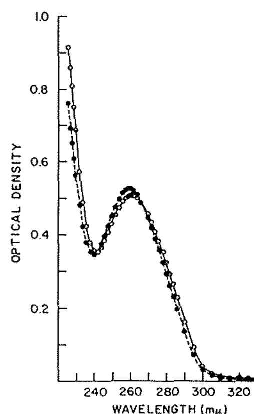


Fig. 2 Ultraviolet spectraof defective particles (○) and normal MS2(●) at pH 7.0

light particles are not infectious (Fig. 1), and in keeping with their low buoyant density, the ratio of RNA to protein being 0.19, compared with 0.29 for normal MS2 prepared in the same way. The ultraviolet spectrum of these particles, shown in Fig. 2, supports these results obtained from chemical analysis. By electron microscopy, the normal and defective particles are indistinguishable (kindly carried out by Dr. E. Moudrianakis). If the protein content of light particles is assumed to be normal, the average RNA content is about two-thirds of normal.

(b) Properties of the RNA of defective particles

The RNA isolated from defective particles is heterogeneous ; the bulk of the RNA had an S_{20} value of 20 in 0.01 *M*-Tris HCl (pH 7.4) and 0.01 *M*-magnesiumacetate, compared with a value of 27 for MS2 RNA. The sedimentation rate of 20*S* is consistent with the presence of a single polynucleotide chain of about two-thirds the normal length. The base composition was not different from normal MS2 RNA (except for 5-fluorouracil). The melting profile was essentially identical with that of intact MS2 RNA containing FU,[11] i.e. the melting temperature (Tm) was 61.5°C and there was about 10% less total hyperchromicity compared with normal MS2 RNA as shown in Fig. 3. The significance of this observation is not known.

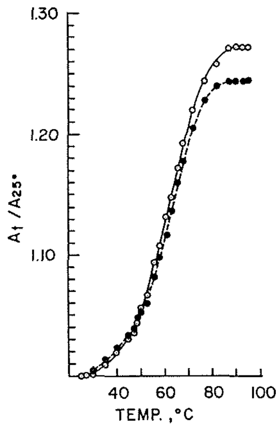


Fig. 3 Melting curves of normal and FU-fragment RNA FU defective phage and normal MS2 prepared in parallel were dialyzed in the same flask against 0.1 *M*-sodium phosphate buffer of pH 6.8. RNA was extracted from each phage preparation and the optical density at 259 mμ determined in a heated-holder at the temperatures indicated. Normal RNA (○): FU-fragment RNA (●).

(c) **RNA fragment as a messenger**.

Although these fragments were not infectious for spheroplasts,[19] they were quite active in directing protein synthesis in cell extracts; in fact they were more active than normal MS2 RNA by a factor of two to three (Fig. 4). It is worth to note that *incorporation into protein of histidine, an amino acid not* found in MS2 coat protein, is stimulated to about the same extent as that of amino acids present in coat protein (proline and phenylalanine).

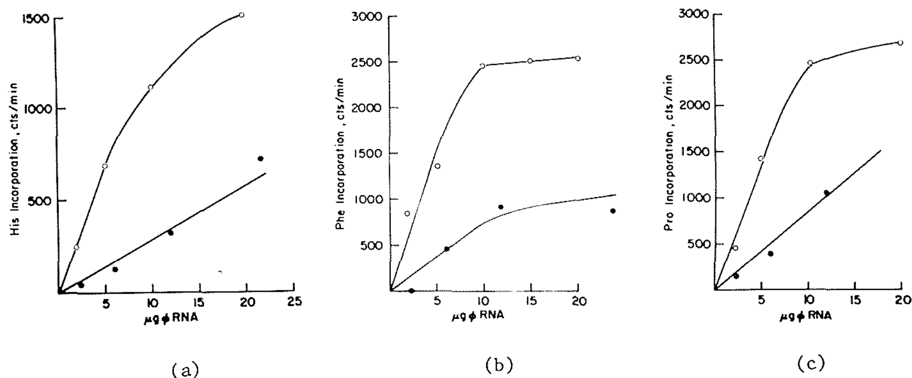


Fig. 4 *In vitro* incorporation into protein of (^{14}C)histidine (a), (^{14}C)phenylalanine (b) and (^{14}C)proline (c) stimulated by RNA from defective particles (○) and from intact MS2 (●). Incubations were carried out as previously described.[12]

That all the messenger activity is associated with the fragment of RNA is shown in Fig. 5, which compares the sedimentation in sucrose and activity of RNA from normal MS2 (Fig. 5 (a)) and from defective particles (Fig. 5 (b)).

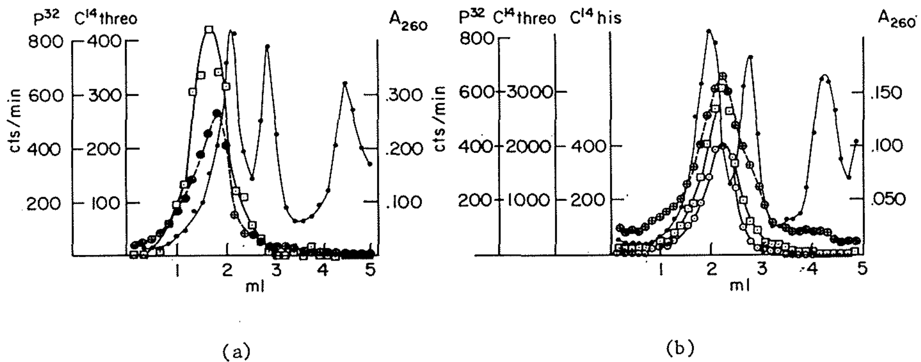


Fig. 5 Sedimentation and messenger activity of RNA from normal MS2 (a) and from defective oarticles (b).

Samples of phage RNA were mixed with (^{32}P)-labelled *E. coli* RNA and centrifuged through a 5 to 20% sucrose gradient at 35,000 rev/min for 4.5 hr in a Spinco SW39 rotor. Each fraction was tested for stimulation of (^{14}C) threonine (□) and (^{14}C) histidine (○). A_{260} (⊕) and ^{32}P marker RNA (●) are also given. The (^{14}C)-histidine incorporation directed by normal RNA is not plotted, but corresponded with the (^{14}C)-threonine peak.

As seen in the Figure, the activity of the RNA fragments for incorporation of both histidine and threonine (present in phage coat) corresponds to the optical density peak. Also evident in the Figure is the enhanced activity of the RNA fragments.

(d) Cistrons of the RNA fragment

Grossly normal coat protein has been identified as one of the products by two methods, fingerprint analysis and Sephadex chromatography. For analysis of the tryptic peptides, the protein products directed by the RNA fragment were labelled with (^{14}C)arginine and (^{14}C)lysine and the products directed by normal RNA were labelled with (^{3}H)arginine and (^{3}H)lysine. Both products were mixed, authentic coat protein added, this mixture was then digested with trypsin

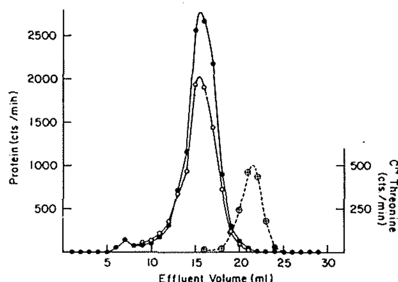
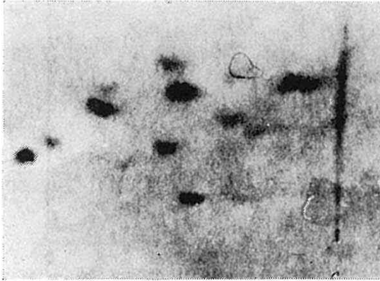


Fig. 6 Sephadex chromatography of threonine-labelled protein, synthesized *in vitro*.

Chromatography was carried out as previously described.[12] ●, (^{3}H) threonine-labelled protein made with normal MS2 RNA: ○, (^{14}C) threonine-labelled protein made with FU-particle RNA; ⊕, free (^{14}C)threonine marker. The dominant peak corresponds to phage coat protein.



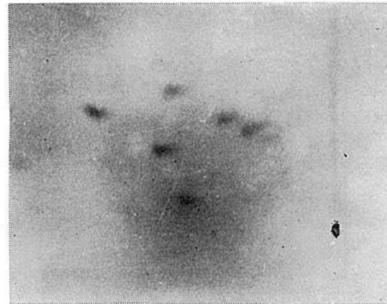


Plate. 1 Fingerprint and radioautogram of (^{14}C)arginine and (^{14}C)lysine-labelled product.

Protein synthesis was carried out as described previously[12] for 30 min in the presence of either (^{14}C)arginine, (^{14}C)lysine and 0.3 mg FU-fragment RNA in 0.5 ml or (^{3}H)arginine, (^{3}H)lysine and 0.3 mg normal MS2 RNA in 0.5 ml. The (^{14}C)-labelled product and the (^{3}H) labelled products were mixed. The protein of the 105,000 g supernatant fraction of the incubation mixture was fingerprinted with carrier coat protein after tryptic digestion as described previously.[12] Left : Ninhydrin-stained paper ; Right : Crresponding radioautogram of (^{14}C)-labelled product, developed after 1 week. The circled spot on the fingerprint denotes a peptide unstained by ninhydrin, but detectable by fluorescence with ultraviolet light.

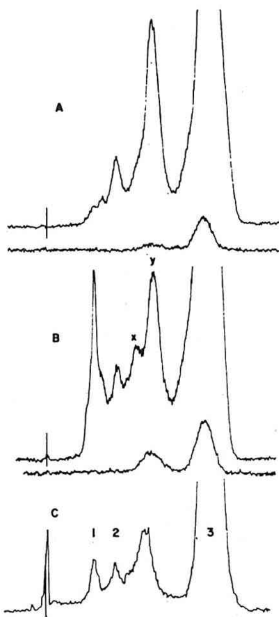


Fig. 7 Microdensitometer tracings of the proteins made in *E. coli* extracts in the presence or absence of MS2 RNA.

Protein synthesis occurred in the presence of (^{14}C) isoleucine, threonine, arginine, valine and lysine. A. 15 minute incubation. Top: with MS2 RNA; Bottom: no MS2 RNA. B. 30 minute incubation. Top : with MS2 RNA; Bottom : no MS2 RNA. C. Phage protein from cells infected with MS2, labelled with (^{14}C) isoleucine, arginine, valine and lysine as described previously.

and the peptides analyzed as described by Nathans.[12] For each peptide, the ^{3}H/^{14}C

ratio of peptides was determined. The fingerprint showed all the soluble coat protein peptides in their usual position and in the same relativeamounts as the peptides of the protein made with normal RNA (Plate 1). For Sephadex chromatography, the two products were similarly labelled with (^{14}C)or (^{3}H)threonine; the mixture treated with 6.5 *M*-guanidine hydrochloride, and chromatographed on Sephadex G200 as previously described.[12] As shown in Fig. 6, the major radioactive peak in each case corresponded to that of authentic coat protein. From these experiments, it has been concluded that the RNA fragment contains the entire cistron for the coat protein.

In order to determine what other cistrons are present, the cell-free products directed by the RNA fragment have been examined by acrylamide gel electrophoresis. In cells infected with MS2 phage in the presence of actinomycin D, three phage specific proteins are detectable by acrylamide gel electrophoresis. In Fig. 7, microdensitometer tracings of radioautograms of polyacrylamide gel are presented. The numbered peaks are phage specific proteins made in the infected cells (Fig. 7 (C)). From the analysis of proteins made by various amber mutants, each peak has been identified; i.e. peak 3 as phage coat protein, peak 2 as maturation protein, and peak 1 as probably the RNA synthetase.[6] It is evident from the tracings (A and B) shown in Fig. 7 that the three phage proteins synthesized in MS2-infected cells are also synthesized in *E. coli* extracts programmed with MS2 RNA, as judged by similarity in electrophoretic mobility (and in the case of peak 3, by analysis of tryptic peptides). As seen in Fig. 7, two other phage-specific protein peaks are present (labelled X and Y). Although these proteins have not been identified, peak X is not present at early times and may be a breakdown product of the "maturation" protein. Peak Y, however, appears early and is therefore not likely to consist only of breakdown products. Whether it consists of precursor polypeptides or is an entirely different phage protein remains to be determined.

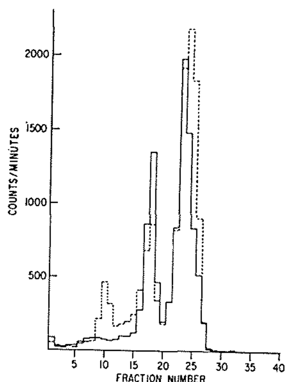


Fig. 8 Electropherogram of proteins made in cell extracts with MS2 RNA (dotted-line) and with FU-fragment RNA (solid line) as messenger.

Proteins were synthesized as described previously[4] for 30 min in the presence of either (^{14}C)arginine, isoleucine, lysine, threonine, valine and FU-fragment RNA or (^{3}H) arginine, isoleucine, lysine, threonine, valine and intact MS2 RNA. The two products were mixed and coelectrophoresed as described in *Methods.* Gels were sliced, and protein was eluted with 0.1% sodium dodecyl sulfate and counted. The first segment includes the origin.

When the cell-free products directed by the FU containing fragment were compared with the protein directed by normal RNA, the results shown in Fig. 8

were obtained. In this experiment, the protein products directed by the RNA fragment were labelled with (^{14}C)threonine and the products directed by normal RNA were labelled with (^{3}H)threonine. Both products were mixed and coelectrophoresed.

As seen in this Figure, normal MS2 RNA directs the synthesis of proteins which yield three main peaks on electrophoresis. The large peak at the right is coat protein, the peak on the left corresponds to peak 1 protein seen in infected cells, tentatively identified as the phage RNA synthetase. The middle peak is thought to be peak Y and, in part, peak X seen in Fig. 7. The FU containing fragment, on the other hand, directs the synthesis of coat protein, the middle protein, but not the peak 1 or RNA synthetase. In either case, the protein corresponding to maturation protein was not detected. This is probably due to reduced resolution when the gel was sliced. In fact, with the same preparation, a peak corresponding to the maturation protein was observed both in the normal products and in the fragment products, where the gels were radioautogrammed. From these results, it has been concluded that the FU fragment makes coat protein and at least a small amount of the protein corresponding to maturation protein but not that corresponding to RNA synthetase. These results also suggest that a specific cistron or cistrons are missing in the FU fragment.

In order to distinguish the effect of FU in the messenger and the fragmentation itself, the products obtained with the whole molecules of RNA containing FU were also examined. The results have not been entirely consistent, but in most experiments, all the normal phage protein products have been detected. In addition, similar absence of the peak 1 protein was found with RNA fragments isolated from amber mutants of the maturation protein[24] and from phage RNA digested with T1 ribonuclease.[22] Therefore, the results obtained with the FU fragment are probably unrelated to the presence of FU.

(e) Determination of the 5′-terminus of RNA fragment

An attempt was made to determine whether the FU fragment was derived from the 5' or 3' end of phage RNA by looking for the presence of nucleoside tetraphosphate after alkaline digestion. Other workers have recently reported the presence of guanosine triphosphate (GTP) at the 5' end of MS2 RNA.[20] When the alkaline hydrolysates of (^{32}P)-labelled FU fragment were electrophoresed, a spot corresponding to the nucleoside tetraphosphate was detected, indicating that the fragment has a 5' nucleoside triphosphate. In addition, the relative chain length of FU fragment, calculated from the ratio of radioactivity of nucleoside tetraphosphate divided by 4 (4 x ^{32}P' s) to that in all the 3' nucleoside monophosphate, was about two-thirds that of MS2 RNA (Table 1). On the basis of these findings in conjunction with the results obtained from the analysis of protein products directed by the fragment, it has been concluded that the FU fragment probably consists of the 5' two-thirds of the phage RNA and that the cistron which codes for the RNA synthetase may be at or near the 3' end of the molecule.

Table 1

Chain length of FU fragment

	cpm*			Relative
	pXppp	Total	Ratio	chain length
27S (MS2)	281	308,000	1,060	4,240
20S (FU)	127	82,700	651	2,610

* (^{32}P)-labelled FU defective phage was made according to the method described previously.[11] (^{32}P)-labelled MS2 phage was similarly prepared without addition of FU. RNA was extracted from purified phage and then purified on sucrose gradient.[22] Alkaline hydrolysates of (^{32}P)-labelled RNA were electrophoresed on Whatman 3MM paper as described in *Methods.* The nucleotide spots, detected from the radioautogram, were cut out and counted.

Discussion

(a) Production of RNA fragment

The mechanism of production of FU-RNA fragment is, of course, relevant to that of formation of defective FU particles. This mechanism is best explained by the observations of Lodish et al.,[7] Heisenberg[8] and Argetsinger and Gussin[9] that a certain class of mutants of the RNA coliphage gives rise in the non-permissive host to RNA deficient particles. In fact, Lodish et al. independently observed that FU mimicked this mutation. The suggestion that defective mutant particles are formed because of non-functional "maturation" protein (or "RNA-protecting" protein) would also explain the formation of defective virus particles in the case of 5-fluorouracil.[19] An alternate possibility is that the FU particle has abnormal coat protein subunits which do not fit as compactly as normal coat, thereby allowing nucleases to degrade the RNA.[21] It is noteworthy that the RNA fragments of phage mutants having an amber mutation in the maturation cistron have S_{20} values of 23 and 17.[22]

The failure of FU particles to adsorb to *E. coli* receptor sites may be due to abnormalities in coat protein or, more likely, to a defect in the protein required for viral "maturation." The normal phage appears to have a small quantity of the latter protein,[4] which may serve as the site of attachment of the phage to the bacterial cell.

(b) Effect of 5-fluorouracil in messenger RNA

As noted previously, about 80% of the uracil was replaced in the FU fragment.[11] In spite of this high degree of replacement, the synthetic phage-specific proteins fractionated normally on Sephadex, and fingerprint analysis of the synthesized coat protein showed a peptide map indistinguishable from that of the coat protein made with normal MS2 RNA as template. These observations are in agreement with those of Grunberg-Manago and Michelson,[23] who failed

to find errors of translation with FU-containing synthetic polynucleotides. Our results, however, do not exclude the possibility that errors in translation result from the presence of FU in messenger RNA, since the method employed would require errors greater than 10% for a given peptides. It is possible that there are minor changes in the amino acid sequence of the coat protein made with the FU fragment as template but not detectable by the fingerprint analysis because of their inconstant occurrence.

(c) RNA fragment as a messenger

The high activity of the FU-RNA fragment as a messenger in directing the synthesis of phage-specific proteins in cell extracts is not fully explainable. It is not likely that the high activity depends on the fragmentation, since the whole molecules of RNA containing FU also manifest similarly high activity as a messenger (Shimura, unpublished observation). It is possible that the high activity of FU-RNA is related to some ambiguous codings due to the presence of FU in the messenger molecules.

(d) Cistrons of RNA fragments

The results obtained in this study strongly indicate that the FU fragment consists of the 5' two-thirds of the phage RNA and that this fragment includes the cistrons which code for the coat protein and for the maturation protein. This fragment messenger does not direct the synthesis of RNA synthetase in cell extracts, suggesting that at least part of the cistron for this protein is missing in the fragment. The simplest interpretation of these observations is that the cistron for the RNA synthetase is located at or near the 3' end of the phage RNA, which has been cleaved from the rest of the molecule in the formation of the FU defective particles. It is of interest that one of the fragments (20*S*) obtained by limited digestion of MS2 RNA with ribonuclease T1 also lacks at least part of the cistron for this enzyme.[22]

From the analysis of other fragments, we have obtained evidence indicating that the cistron for the phage coat protein is located nearest to the 5' end of the phage RNA molecule.[22, 24] These results are consistent with the observation made by Eggen et al.,[6] which showed that the synthesis of the three phage proteins in cell free extract is sequential, in the order coat protein, the protein corresponding to maturation protein and finally the protein corresponding to RNA synthetase.

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