*CONTROL OF TRANSLATION OF MS2 RNA CISTRONS BY MS2 COAT PROTEIN**

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The RNA isolated from MS2 phage forms two types of stable complexes with MS2 coat protein *in vitro*.[1] The first type, termed "complex I," is apparently a complex which contains one molecule of MS2 RNA and a few molecules of MS2 coat protein. The second type, termed "complex II," contains more coat protein per molecule of MS2 RNA and looks very similar to an MS2 virion under the electron microscope. Complex II particles, however, are not infectious.

A complex similar to complex I has also been observed by Capecchi and Gussin[2] during the *in vitro* synthesis of coat protein under the direction of R17 RNA. They suggested that the formation of this complex might be the initial step in the maturation of the virus.

We thought, however, that complex I might serve as a device in the regulation of viral RNA translation. Previously, Ohtaka and Spiegelman[3] suggested that the infection of cells with MS2 phages must induce some mechanism to ensure that the cistron for coat protein is translated more often than other cistrons. Their suggestion was based on the theoretical consideration that a much larger quantity of coat protein is needed than of other proteins. We postulated that MS2 coat protein itself might play a role in this mechanism by forming a complex with MS2 RNA, complex I, thereby blocking translation of the cistrons for noncoat proteins. The results presented in this paper support our hypothesis.

General Procedure.—We have studied the possibility outlined above by using MS2 RNA and an *in vitro* protein-synthesizing system of *Escherichia coli*, host bacterium for MS2. We have assumed that (1) the incorporation of histidine measures the synthesis of MS2-specific proteins other than coat protein since this amino acid is absent from the coat protein of MS2; and (2) the incorporation of another amino acid such as phenylalanine measures the synthesis of total MS2-specific proteins. To examine the effect of coat protein on the *in vitro* translation of MS2 RNA, complex I (a preparation of MS2 RNA incubated with MS2 coat protein) was added to the protein-synthesizing system in place of MS2 RNA.

Materials and Methods.—MS2 RNA, MS2 coat protein, and H^3-phenylalanine-labeled MS2 virus were prepared and their molar concentrations determined as described previously.[1] The protein solution was used within one day of its preparation to ensure reproducible results. Tobacco mosaic virus (TMV) RNA was isolated with phenol from TMV which was kindly provided by Dr. H. Fraenkel-Conrat. Polyuridylic acid (poly U) was purchased from Miles Chemical Company. C^{14}-L-amino acids (alanine, 117 mc/mmole; histidine, 247 mc/mmole; leucine, 275 mc/mmole; phenylalanine, 367 mc/mmole) were purchased from New England Nuclear Corporation.

Complex I formation: Complex I was formed by mixing MS2 RNA and MS2 coat protein in 0.1 *M* tris-HCl buffer, pH 7.0, at the protein:RNA ratio described in each experiment. To avoid precipitation of the protein, the RNA and buffer were first mixed, and then protein was added to the RNA-buffer mixture. In early experiments the mixture was incubated at 37°C for 60 min to ensure the formation of complex I, but omission of the incubation in later experiments seemed to have no effect on subsequent amino acid incorporation.

In vitro amino acid incorporation: Cells of *E. coli* Q13 (kindly provided by Dr. S. Spiegelman) were used to prepare cell-free extracts. The cells were ground with alumina and extracted with 0.01 *M* tris-HCl, pH 7.8, containing 0.01 *M* magnesium acetate, 0.06 *M* KCl, 0.006 *M* mercaptoethanol, and 5 μg/ml of DNase. The extract was centrifuged according to the method of Nirenberg and Matthaei[4] to obtain the 30,000 *g* supernatant fraction, termed S30. The S30 fraction was incubated at 35°C for 30 min following the method of Slater and Spiegelman[5] and stored at −70°C. One volume of MS2 RNA solution, which had been incubated in 0.1 *M* tris-HCl, pH 7.0, with or without MS2 coat protein, was added to 2 volumes of an incorporation mixture to initiate the amino acid incorporation. The final reaction mixture, usually 0.3 or 1.0 ml, contained per ml: 50 μmoles tris-HCl, pH 7.5; 12.5 μmoles magnesium acetate; 30 μmoles KCl; 30 μmoles NH_4Cl; 5 μmoles mercaptoethanol; 1 μmole ATP; 0.05 μmole GTP; 5 μmoles phosphoenolpyruvate; 20 μg pyruvate kinase (Sigma Chemical Company); 100 μg stripped transfer RNA from *E. coli* K12 (General Biochemicals Inc.); 0.05 μmole each of 20 L-amino acids except the one added as the C^{14}-amino acid. The reaction mixture also contained 1.3 mg (protein) of incubated S30 fraction and 1 μc of the desired C^{14}-L-amino acid per ml. The amounts of MS2 RNA and coat protein added to the system are given in Tables 1 and 2. The reaction mixture was incubated at 35°C for 40 min. At the end of this period, an aliquot, 0.1 or 0.3 ml, was removed to determine the radioactivity of hot trichloroacetic acid (TCA)-insoluble material, and the rest of the reaction mixture was frozen at −70°C for further analysis.

Chromatography on a Sephadex G200 column: A modified procedure from that originally described by Nathans[6] was used to analyze the products of the *in vitro* amino acid-incorporating system. The *in vitro* products (0.025–0.1 ml of the reaction mixture) and about 5 μg (0.01 ml) of H^3-phenylalanine-labeled MS2 virus were placed in a small test tube and made up to 0.5 ml with 6.5 *M* guanidine-HCl solution containing 0.14 *M* mercaptoethanol, 0.001 *M* EDTA, and 0.05 *M* tris, pH 8.5. After a brief flushing with nitrogen, the tube was tightly sealed and incubated at 45°C for 5–6 hr. Blue Dextran (Pharmacia, mol wt = 2×10^6), 0.05 ml of 0.5% solution in the same guanidine-HCl solution, was added to the incubated mixture. Two-tenths ml of the mixture was placed on a Sephadex G200 column (1 × 28 cm) which had been equilibrated with the guanidine-HCl solution. The same guanidine-HCl solution was also used for elution. The flow rate was 2–3 ml per hour, and 0.5-ml fractions were collected. The hot TCA-insoluble material in each fraction was collected and washed on a Millipore filter, and the radioactivity of H^3 and C^{14} was counted for at least 10 min by a Packard liquid scintillation counter.

Results.—In vitro amino acid incorporation: Preparations of MS2 RNA previously incubated with or without MS2 coat protein were used to stimulate *in vitro* amino acid incorporation. MS2 RNA preparations which had been incubated with varying concentrations of MS2 coat protein were used as complex I, since no technique was available to separate this complex from MS2 RNA. At the higher protein:RNA ratios used in this experiment, preparations of complex I might have contained a small amount of complex II.[1] However, when partially purified complex II was tested, it had no stimulatory effect on *in vitro* amino acid incorporation in the same cell-free system.

As shown in Table 1, when MS2 RNA had been incubated with MS2 coat protein, the subsequent *in vitro* incorporation of C^{14}-histidine (presumably the synthesis of MS2-specific, noncoat proteins) was more strongly inhibited than the incorporation of C^{14}-phenylalanine (presumably the synthesis of total MS2-specific proteins). In several similar experiments, the inhibition of histidine incorporation ranged from 71 to 77 per cent while the inhibition of phenylalanine incorporation ranged from 19 to 36 per cent at protein:RNA ratios between 20:1 and 48:1. These results are consistent with the hypothesis that MS2 coat protein, by virtue of its association with MS2 RNA, i.e., complex I formation, inhibits translation of the cistrons for noncoat proteins in MS2 RNA.

The effect of incubation of MS2 RNA with MS2 coat protein was also tested

TABLE 1

INCORPORATION OF C^{14}-PHENYLALANINE AND C^{14}-HISTIDINE INTO HOT TCA-INSOLUBLE MATERIAL DIRECTED BY MS2 RNA AND COMPLEX I

MS2 RNA used (μg)	MS2 protein used (μg)	Molar ratio of protein to RNA	C^{14}-Phenylalanine Incorporation		C^{14}-Histidine Incorporation	
			Cpm incorp.*	Control (%)	Cpm incorp.*	Control (%)
50.3	0 (control)	0	28,649	100	7,016	100
50.3	1.6	2.0	29,870	104	4,588	65
50.3	8.0	9.8	29,322	102	2,533	36
50.3	32.0	39.2	22,100	77	1,763	25

Complex I was formed with the specified amount of MS2 RNA and MS2 protein at 37°C for 60 min. The conditions for complex I formation and *in vitro* amino acid incorporation are described in *Materials and Methods*. A total volume of 0.3 ml of the incubation mixture per sample was used for incubation and for the counting of hot TCA-insoluble material.

* Average of duplicate samples. The endogenous incorporations (no RNA addition) of 3,697 cpm for phenylalanine and 753 cpm for histidine were subtracted. Coat protein showed no effect on the endogenous incorporations.

for the subsequent *in vitro* incorporation of C^{14}-leucine and C^{14}-alanine. The incorporation of these amino acids was inhibited to a similar extent as that of phenylalanine. In parallel experiments, the incorporation of histidine was always inhibited to a greater extent than that of the other amino acids tested.

The inhibitory effect caused by MS2 coat protein on amino acid incorporation was specific to MS2 RNA. This protein did not alter the amino acid incorporation stimulated by TMV RNA or poly U in the same system. These results are shown in Table 2. MS2 coat protein incubated with TMV RNA[7] or poly U[1] failed to form an RNA-protein complex similar to complex I. These observations suggest that the inhibitory effect of MS2 coat protein is exerted directly on MS2 RNA rather than on other components of the amino acid-incorporating system.

Chromatography on a Sephadex G200 column: The products directed *in vitro* by MS2 RNA or by complex I were analyzed by chromatography on a Sephadex G200 column in 6.5 *M* guanidine-HCl. When the original procedure described by Nathans[6] was closely followed, the results obtained were very similar to those obtained by Nathans.[6] However, under his conditions, a small but significant

TABLE 2

THE EFFECT OF MS2 COAT PROTEIN ON THE INCORPORATION OF C^{14}-PHENYLALANINE AND C^{14}-HISTIDINE DIRECTED BY MS2, RNA, TMV RNA, AND POLY U

RNA used (μg)	MS2 protein used (μg)	Molar ratio of protein to RNA	C^{14}-Phenylalanine Incorporation		C^{14}-Histidine Incorporation	
			Cpm incorp.*	Control (%)	Cpm incorp.*	Control (%)
MS2 RNA						
50.3	0 (control)	0	17,075	100	3,411	100
50.3	2.0	2.4	12,957	76	2,881	84
50.3	9.8	12.0	12,500	73	1,784	52
50.3	39.2	48	10,939	64	983	29
TMV RNA						
50.5	0 (control)	0	5,927	100	3,384	100
50.5	2.0	4.8	5,954	100	3,698	109
50.5	9.8	23.2	6,106	103	3,490	103
50.5	39.2	92	5,625	95	2,826	84
Poly U						
26.8	0 (control)	—	122,304	100	—	—
26.8	2.0	—	121,673	99	—	—
26.8	9.8	—	125,445	102	—	—
26.8	39.2	—	125,570	103	—	—

RNA was incubated with MS2 protein as specified at 0°C for about 60 min before addition to the amino acid-incorporating system. Other conditions were the same as in Table 1.

* Average of duplicate samples. The endogenous incorporations (no RNA addition) of 2,399 cpm for phenylalanine and 1,174 cpm for histidine were subtracted.

amount of coat protein, derived from MS2 phage, frequently appeared in a region between void volume and the major peak position of coat protein. This made the interpretation of the data difficult in the present experiments. The difficulty was solved, however, when 0.14 *M* mercaptoethanol and 0.001 *M* EDTA were included in the 6.5 *M* guanidine-HCl solution throughout the Sephadex G200 column chromatography. These conditions were used in order to disrupt the cross-linking of peptides by reducing disulfide bonds. Under the modified conditions, the appearance of a portion of MS2 coat protein in a region of higher molecular weight in the Sephadex G200 column was virtually eliminated. This made it possible to analyze the materials eluted from the column in a region between void volume and coat protein. Figure 1 shows the results of a typical experiment obtained by the modified procedure.

The chromatographic profile of C^{14}-phenylalanine-labeled products (presumably total MS2-specific proteins) formed *in vitro* in the presence of MS2 RNA showed a major peak which coincided with the peak of H^3-phenylalanine-labeled MS2 coat protein added as a marker (Fig. 1*a*). However, a noticeable amount of radioactive materials also eluted both before and after the peak position of coat protein. The amount of materials which eluted before coat protein was significantly reduced when complex I was used to direct phenylalanine incorporation in place of MS2 RNA (Fig. 1*b*). On the other hand, the amount of material which appeared in the major radioactive peak, coinciding with the peak of coat protein, was only slightly reduced.

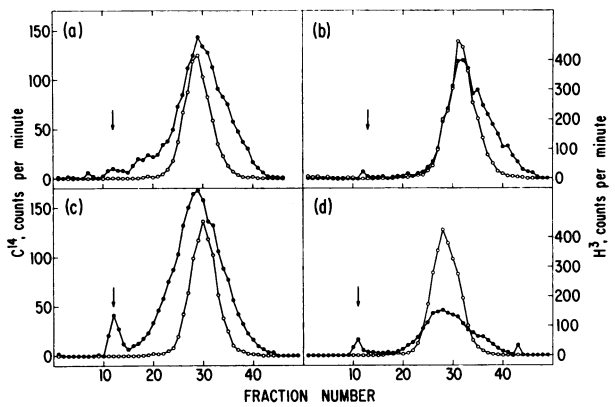

FIG. 1.—Sephadex G200 chromatography with 6.5 *M* guanidine-HCl containing 0.14 *M* mercaptoethanol, 0.001 *M* EDTA, and 0.05 *M* tris, pH 8.5: An aliquot of the products directed *in vitro* by MS2 RNA or complex I was mixed with H^3-phenylalanine-labeled authentic MS2 virus. The mixture was incubated in guanidine-mercaptoethanol-EDTA-tris buffer and chromatographed as described in *Materials and Methods*. In this experiment, complex I was prepared with a 20-fold excess (in molar concentration) of MS2 protein. The amounts of C^{14}-phenylalanine and C^{14}-histidine incorporated under the direction of complex I were 73 and 26%, respectively, as compared to those obtained under the direction of MS2 RNA. Four times greater amount of C^{14}-histidine-labeled products was used than C^{14}-phenylalanine-labeled products. The arrow in the figure indicates the position of the elution of Blue Dextran (mol wt = 2×10^6) added as an internal marker for each run. (*a*) C^{14}-phenylalanine-labeled products directed by MS2 RNA, (*b*) C^{14}-phenylalanine-labeled products directed by complex I, (*c*) C^{14}-histidine-labeled products directed by MS2 RNA, (*d*) C^{14}-histidine-labeled products directed by complex I. *Closed circles:* C^{14}-labeled products of *in vitro* amino acid-incorporating system. *Open circles:* H^3-labeled MS2 coat protein from MS2 virus.

The chromatographic profile of C^{14}-histidine-labeled products (presumably MS2-specific, noncoat proteins) formed *in vitro* showed a more marked difference depending on whether their synthesis was directed by MS2 RNA or complex I. As shown in Figure 1*c* and *d*, the amount of materials appearing in a sharp peak at the void volume region and in a broad peak overlapping the peak of coat protein was greatly reduced when complex I was used to direct histidine incorporation in place of MS2 RNA.

Granted that all the histidine-containing materials are MS2-specific proteins other than coat protein, then these results are consistent with the hypothesis that MS2 coat protein functions in a regulatory mechanism which shuts off translation of the MS2 RNA cistrons for noncoat proteins.

Discussion.—In order to examine the effect of MS2 coat protein on the *in vitro* synthesis of proteins directed by MS2 RNA, it is first necessary to discuss the nature of the products synthesized. The results obtained in this study, involving total amino acid incorporation and Sephadex G200 chromatography of the *in vitro* products, obviously are not sufficient alone to identify the products. Correlation of these results with evidence on similar systems provided by other laboratories, however, makes it seem reasonable to conclude that MS2 coat protein and MS2-specific, noncoat proteins are synthesized in our *in vitro* amino acid-incorporating system.

The argument which supports the notion that MS2 coat protein was synthesized in the *in vitro* system is as follows: Nathans[6] showed that "fingerprints" of tryptic digests of the products formed *in vitro* by stimulation with MS2 RNA matched well those derived from MS2 coat protein. He also showed that a large part of the *in vitro* products cochromatographed with MS2 coat protein on a Sephadex G200 column. A major portion of our products also cochromatographed with MS2 coat protein when analyzed by Nathan's method.[6] Capecchi and Gussin[2] and Capecchi[8] showed that R17 coat protein synthesized *in vitro* under the direction of R17 RNA sedimented together with R17 RNA after centrifugation in sucrose gradients as a result of complex formation. In preliminary experiments, we have also found that about 10 per cent of the C^{14}-phenylalanine-labeled products sedimented with MS2 RNA in sucrose density gradient centrifugation. We believe this fraction contains complex I which is formed from added MS2 RNA and newly synthesized MS2 coat protein. The percentage of newly synthesized coat protein found by this technique is probably low because complex formation is an inefficient process.

The notion that the histidine-containing products synthesized in our *in vitro* system were MS2-specific, noncoat proteins is supported by the following facts. Capecchi[8] showed that a histidine-containing peptide, formed in his *in vitro* protein-synthesizing system directed by R17 RNA, sedimented at 20*S* in a sucrose gradient. This material, after oxidation with performic acid to disrupt the cross-linking of peptides by disulfide bonds, cochromatographed with Blue Dextran on a Sephadex G200 column. He identified this peptide as an R17-specific RNA synthetase by using a mutant of R17 defective in the production of the RNA synthetase. In place of oxidation with performic acid, we reduced unfractionated *in vitro* products with mercaptoethanol to disrupt the cross-linking of peptides by disulfide bonds. After this treatment, we found a sharp peak of histidine-containing material which

cochromatographed with Blue Dextran on a Sephadex G200 column. Since MS2 and R17 are very closely related to each other,[9, 10] this material may be the MS2-specific RNA synthetase.

A broad peak of histidine-containing materials which overlapped the area of MS2 coat protein might contain more than one peptide. Although we do not know their identity at present, we suspect that they are also MS2-specific, noncoat proteins.

Granted that both intact MS2 coat protein and MS2-specific noncoat proteins are synthesized in our *in vitro* amino acid-incorporating system, we now compare the products formed *in vitro* under the direction of free MS2 RNA and of the complex formed between MS2 RNA and MS2 protein, i.e., complex I.

When complex I was used in place of MS2 RNA to stimulate *in vitro* amino acid incorporation, a marked reduction in histidine incorporation and a moderate reduction in phenylalanine incorporation were found (Table 1). Since histidine is absent and phenylalanine is present in MS2 coat protein, these results support the hypothesis that complex I directs the preferential synthesis of coat protein.

The results obtained with Sephadex G200 chromatography are also consistent with this hypothesis: The amounts of all products, except C^{14}-phenylalanine-labeled material which cochromatographed with authentic coat protein, were greatly reduced when complex I was used in place of MS2 RNA. The small reduction (17%) of phenylalanine-labeled material at the peak position of coat protein may be explained by the reduction of histidine-containing noncoat proteins whose chromatographic position coincides with that of coat protein (Fig. 1*c* and *d*).

The identity of the material in the shoulder which trailed the peak of coat protein, in the C^{14}-phenylalanine-labeled products directed by complex I, is unknown (Fig. 1*b*). Since only a small amount of labeled material was found in the corresponding region of histidine-labeled products (Fig. 1*d*), this material may be considered as fragments of coat protein. Also, the nature of complex I-directed histidine-labeled material which coincided with coat protein in Sephadex chromatography (Fig. 1*d*) is unknown. This material may be the result of incomplete inhibition of the translation of noncoat protein cistrons by the coat protein of complex I. It is also possible that this material is the product whose synthesis is not subject to the postulated control mechanism.

The suggested role played by complex I in the control of translation of MS2 RNA, as presented above, is based on our *in vitro* experiments. It is tempting to propose that the same control mechanism may also exist in MS2-infected cells.

A few reports dealing with the synthesis of virus-specific proteins in cells infected with small RNA viruses support this proposition. In MS2-infected *E. coli* cells[11] as well as in polio-infected HeLa cells,[12] synthesis of coat protein(s) begins at an early stage of infection. The ratio of coat protein to other virus-specific proteins is low early in the infectious process, but it later becomes progressively higher. It appears, therefore, that the coat protein of small RNA viruses is synthesized from the beginning of infection and the synthesis continues throughout. In f2 phage-infected cells, the amount of virus-specific RNA synthetase levels off about halfway through the infectious process.[13] However, in some strains of nonpermissive cells infected with amber mutant phages unable to direct the synthesis of coat protein such as Su-3 or Su-11, the amount of virus-specific RNA synthetase

does not level off but continues to increase. This increase may be explained by the lack of complex I formation in these coat-protein mutants.

Summary.—In a cell-free, protein-synthesizing system prepared from *Escherichia coli*, RNA isolated from MS2 phage directs the synthesis of MS2 coat protein which lacks histidine as well as several other proteins which contain histidine. When MS2 RNA is incubated with a small amount of MS2 coat protein before being added to the *in vitro* protein-synthesizing system, the synthesis of histidine-containing proteins is greatly reduced while the synthesis of coat protein is little affected. It is proposed that the formation of a complex between MS2 RNA and a small number of MS2 coat protein molecules, complex I, is a regulatory mechanism which inhibits the translation of cistrons for noncoat proteins. Such a mechanism would allow the preferential synthesis of coat protein in MS2-infected cells.

Note added in proof: A part of the present experiments has appeared in *Federation Proc.*, **26**, 611 (1967). K. Eggen and D. Nathans reported a similar observation (*Federation Proc.*, **26**, 449 (1967)). On the basis of studies on conditional lethal mutants of f2, H. F. Lodish and N. D. Zinder have suggested that f2 coat protein acts, directly or indirectly, as the "repressor" of phage enzyme synthesis (*J. Mol. Biol.*, **19**, 333 (1966)).

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