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*REPLICATION OF VIRAL RNA, VII. FURTHER STUDIES ON THE ENZYMATIC REPLICATION OF MS2 RNA**

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Infection of *Escherichia coli* with RNA[1]-containing phages such as MS2 or f2 leads to the formation of one or more RNA-synthesizing enzymes.[2-4] The available evidence suggests that one of these, RNA synthetase,[2] is associated with a double-stranded RNA template, the replicative form of MS2 RNA, and synthesizes RNA from ribonucleoside triphosphates with no requirement for an added primer. When isolated by phenol extraction, about half of the RNA synthesized is present as double-stranded RNA and is resistant to RNAase.[5] The resistant product has the properties of the replicative form of MS2 RNA.[6, 7] The remainder of the synthesized RNA is RNAase-sensitive and therefore presumably single-stranded. Specific annealing assays showed that the bulk of the radioactivity incorporated into the duplex, on incubation of the partially purified enzyme with labeled nucleoside triphosphates, consists of parental type, "plus" MS2 RNA strands.[7] Thus far, the identity of the single-stranded RNA had not been ascertained. It has now been shown that this RNA is also MS2 RNA of the parental type.

To determine the identity of the single-stranded RNA the C^{14}-labeled synthetase product was annealed with a large excess of heat-denatured, nonlabeled (natural) replicative form of MS2 RNA. Nearly all the C^{14}-labeled RNA became RNAase-resistant (i.e., double-stranded), showing that the single-stranded RNA in the product consisted predominantly of either "plus" or "minus" MS2 RNA strands or of a

mixture of both. Upon thermal denaturation and reannealing of this double-stranded material, specifically in the presence of an excess of nonlabeled MS2 RNA ("plus" strands), nearly 90 per cent of the radioactivity was displaced from the duplex and became RNAase-sensitive, showing that the radioactivity was in MS2 "plus" strands. Thus, most of the RNA formed by RNA synthetase consists of parental-type "plus" strands of MS2 RNA.

Preparations.—MS2 RNA and P^{32}-labeled MS2 RNA (specific activity, about 10,000 cpm/μg) were prepared as described earlier.[6] The replicative form of MS2 RNA was prepared by the method outlined by Langridge *et al.*[8] to be described in detail elsewhere.[9] RNA synthetase (step 4, specific activity 1.0) was purified following the published procedure.[7] Radioactive synthetase product was prepared, as previously described,[7] from UTP-C^{14} (specific radioactivity 62,000 dpm/mμmole), CTP-C^{14} (specific radioactivity 22,600 dpm/mμmole), and nonlabeled ATP and GTP, omitting Mn^{++} from the reaction mixture. The incubation was carried out for 3 min at 34°. The product obtained by phenol extraction and precipitation with ethanol was dissolved in 2 ml of 0.1 × SSC (A_{260}, 47/ml). The bulk of the absorbance was due to viral RNA, replicative form, and predominantly to ribosomal RNA from the enzyme preparation. The radioactivity of the product (655,000 dpm/ml) corresponded to about 10 μg/ml of newly synthesized RNA; 58% was resistant to RNAase. *E. coli* ribosomal RNA was prepared according to Stanley.[10] For control experiments, C^{14}-labeled product of the reaction catalyzed by crude DNA-dependent RNA nucleotidyl transferase (RNA polymerase) was prepared as follows: 2.6 gm of frozen *E. coli* B was ground in a mortar with an equal weight of alumina; 5 ml of a buffer containing 10 mM Tris-HCl, pH 8, 1 mM EDTA, 3 mM $MgCl_2$, and 5 mM mercaptoethylamine was added and the suspension centrifuged 15 min at 27,000 *g*. The supernatant was decanted and recentrifuged as above. The crude extract contained 10 mg of protein/ml and incorporated 7 mμmoles of UMP/mg of protein, in 5 min at 37°, using the same assay mixture as for synthetase[2] but without DNAase and Mn^{++}. No DNA was added. In the presence of DNAase, the incorporation was reduced to less than 1%. C^{14}-UMP-labeled product was prepared as described for the RNA synthetase product except that DNAase was added only at the end of the incubation. The final product, in 0.5 ml of 0.1 × SSC, had 37 A_{260} units and a radioactivity of 940,000 dpm, corresponding to about 20 μg of newly synthesized RNA. Less than 1.0% of the radioactive product was resistant to RNAase.

Other preparations were as described in earlier publications.[6, 7]

Methods.—Determination of absorbance: All absorbances were determined in 0.05 *M* Tris-HCl buffer, pH 7.6 (cell light path, 1.0 cm). For MS2 RNA and ribosomal RNA,[10] the extinction coefficient used was 25.0 mg^{-1} ml, for the replicative form of MS2,[9] 21.0 mg^{-1} ml.

Determination of acid-insoluble radioactivity: To the radioactive RNA sample in 2 ml SSC was added 0.1 ml of 0.1% bovine serum albumin and 0.2 ml of 60% trichloroacetic acid. The mixture was allowed to stand for 10–30 min at 0° and the precipitate was collected on a Millipore HA filter (0.45-μ pore size). The filter was attached to a glass needle, dried under an infrared lamp, and placed in a scintillation vial. Liquifluor diluted 1:25 with toluene was used as scintillation fluid. Radioactivities were measured in a Packard Tricarb scintillation spectrometer. C^{14} was counted with 38% efficiency at 9% gain, with window settings 50–400; P^{32} at 0.7% gain, and window settings 110–1000. The C^{14}-channel registered 5.1% of the radioactivity in the P^{32}-channel. Of each isotope 3000 cpm were usually counted.

RNAase resistance: The sample was diluted with sodium chloride–sodium citrate solution to a volume of 2.0 ml and a final concentration of 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7. After adding RNAase A (50 μg/ml), the solution was incubated for 30 min at 25°. Acid-insoluble radioactivity was determined as described above. Identical samples, treated exactly as described above, but not incubated with RNAase, were used as controls.

Annealing assays: Annealing tubes (length, 10 cm) were prepared from glass tubing (wall thickness, 1 mm; diameter, 7 mm) previously boiled in 0.01 *M* EDTA and washed thoroughly with distilled water. The samples were pipetted into the tubes and SSC was added so that each contained a total of 1.5 μmoles of sodium chloride and 0.15 μmole of sodium citrate. The samples were taken to dryness over P_2O_5 in a vacuum desiccator at 12 mm Hg. Water (0.04 ml) was added to each tube and the contents were mixed on a Vortex mixer. The tubes were sealed, heated for 3

min in an oil bath at 120°, and kept for 60 min at 85°. The contents of the tubes were quantitatively transferred to test tubes with 2 ml of SSC and the RNAase resistance was assayed as described above. All determinations were carried out in duplicate.

Results.—Thermal denaturation and annealing with replicative form: In a control experiment P^{32}-labeled MS2 RNA was mixed with the C^{14}-labeled product of DNA-primed RNA nucleotidyl transferase and the mixture was subjected to thermal denaturation and reannealing, as described in the *Methods* section, in the presence of increasing amounts of unlabeled replicative form of MS2 RNA. The amount of P^{32}- or C^{14}-radioactivity rendered RNAase-resistant at various concentrations of replicative form was measured. Figure 1, curve *1*, shows that nearly 85 per cent of the P^{32}-labeled MS2 RNA became RNAase-resistant at the highest concentrations of replicative form,[11] whereas the C^{14}-labeled RNA nucleotidyl transferase product (curve *2*) remained RNAase-sensitive, i.e., failed to anneal. The specificity of the annealing reaction is further borne out by the experiment shown in the inset to Figure 1. There was no significant annealing of either P^{32}- or C^{14}-labeled RNA when *E. coli* ribosomal RNA was substituted for replicative form.

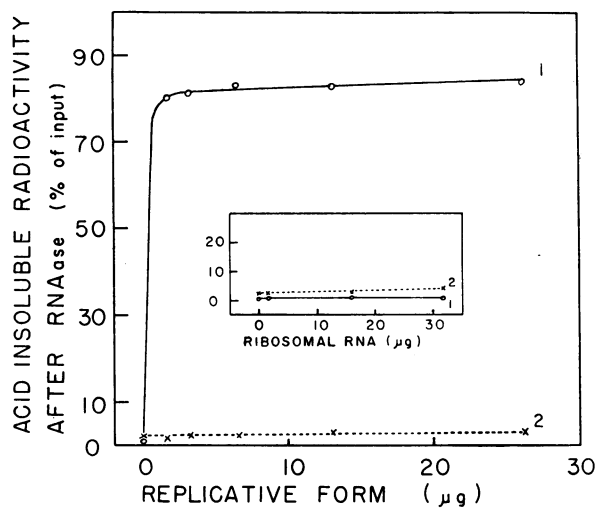

FIG. 1.—Annealing assay of radioactive RNA with thermally denatured replicative form of MS2 RNA. Each sample contained 330 cpm of P^{32}-labeled MS2 RNA (0.07 μg), 430 cpm of C^{14}-labeled product of DNA-primed RNA nucleotidyl transferase (0.046 A_{260} units), and replicative form as indicated, in 0.04 ml of 2.5 × SSC. The annealing assay was carried out as described in the *Methods* section. A nonheated sample had the same amount of acid-insoluble radioactivity as a heated sample. The RNAase resistance before heating was 0.3% for P^{32} and 0.7% for C^{14} radioactivity. All determinations were carried out in duplicate. Inset, control using *E. coli* ribosomal RNA instead of MS2 replicative form. Curve *1* (—O—O—O—), P^{32}-labeled MS2 RNA; curve *2* (-x-x-x-), C^{14}-labeled product of DNA-primed RNA nucleotidyl transferase reaction.

Thermal denaturation and annealing of C^{14}-labeled synthetase product and unlabeled replicative form was carried out in the presence of minute amounts of P^{32}-labeled MS2 RNA as an internal standard. As shown in Figure 2, thermal denaturation and reannealing without addition of replicative form gave a product in which about 20 per cent of both P^{32} and C^{14} radioactivity was RNAase-resistant. The decrease in RNAase resistance of the C^{14}-synthetase product from the initial value of 58 per cent to about 20 per cent may be due in part to incomplete reannealing but mainly to dilution

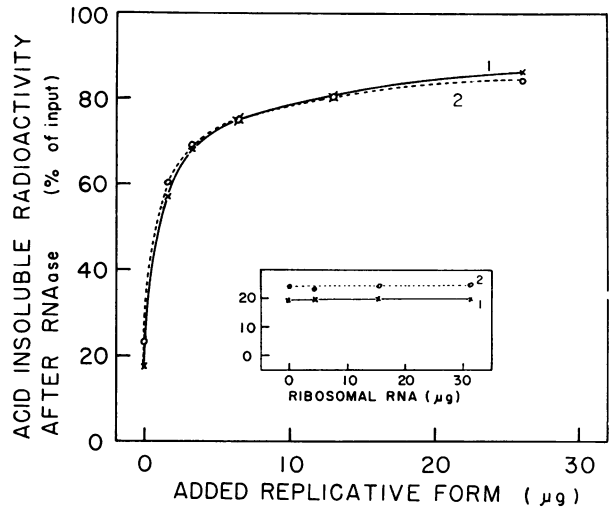

FIG 2.—Annealing assay of thermally denatured C^{14}-labeled synthetase product (and P^{32}-MS2 RNA as internal standard) with replicative form of MS2 RNA. Each sample contained 350 cpm of P^{32}-labeled MS2 RNA (0.085 μg), 250 cpm of C^{14}-labeled synthetase product (0.047 A_{260} units), and replicative form as indicated, in 0.04 ml of 2.5 × SSC. A nonheated sample had the same amount of acid-insoluble radioactivity as a heated sample. The RNAase resistance before heating was 0.6% for P^{32} and 58% for C^{14} radioactivity. All determinations were carried out in duplicate. Inset, control using *E. coli* ribosomal RNA instead of replicative form. Curve *1* (-x-x-x-), P^{32} label; curve *2* (-o-o-o-), C^{14} label.

of C^{14}-labeled "plus" strands by nonlabeled MS2 RNA "plus" strands. The latter were originally present in the synthetase preparation mostly as single-stranded viral RNA and to a lesser extent as replicative form. The conversion of some of the P^{32} radioactivity from RNAase sensitivity to resistance is due to annealing with nonlabeled "minus" strands arising through thermal denaturation from endogenous replicative form. Figure 2 shows further that both P^{32} (MS2 RNA) and C^{14} (synthetase product) radioactivity became RNAase-resistant to the same extent on addition of increasing amounts of replicative form, stressing the similarity of the two RNA's. The inset (Fig. 2) is a control experiment showing the lack of annealing upon substitution of ribosomal RNA for MS2 replicative form.

Specific dilution test of annealed product: The specific dilution test[7] was performed on RNAase-resistant material prepared by annealing C^{14}-synthetase product and P^{32}-MS2 RNA (the latter added as an internal standard) with a large excess of denatured, nonlabeled replicative form. In this preparation about 90 per cent of both the P^{32}- and C^{14}-labeled RNA became resistant to RNAase. As shown in Figure 3, thermal denaturation and reannealing in the presence of large amounts of unlabeled MS2 RNA displaced nearly all of the P^{32} and most of the C^{14} radioactivity. The difference of about 10 per cent may be due to the presence of C^{14}-labeled, i.e., enzymatically synthesized, "minus" strands in the synthetase product. Figure 3 also illustrates the absence of significant displacement of radioactivity by ribosomal RNA. These results show that most of the C^{14}-labeled RNA produced by RNA synthetase, whether as single strands or in the replicative duplex, consists of "plus" MS2 RNA strands of the parental type.

Discussion.—The annealing reactions can be expressed by equations which, on

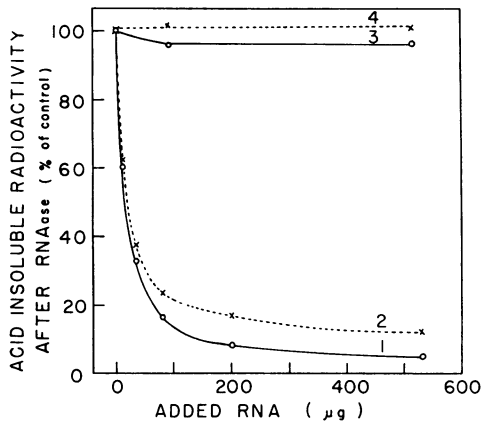

FIG. 3.—Identification of product of synthetase reaction as MS2 RNA of the parental type (specific dilution test). The annealed sample used in the test was prepared as follows: all glassware was flamed or treated with 20% H_2O_2 prior to use, to destroy RNAase. One mg of replicative form in 3 ml of SSC was diluted into 300 ml of boiling distilled water and kept at 100° for 5 min. The solution was cooled to 75° and 3.84 μg of P^{32}-labeled MS2 RNA and 0.03 ml of C^{14}-labeled synthetase product (see *Preparations*) were added. The mixture was frozen, lyophilized (this step led to some loss of material), and dissolved in 0.8 ml distilled water. After 1 hr at 85°, it was diluted to 1.5 ml. The acid-insoluble radioactivity of the solution was 9780 cpm of P^{32} and 4250 cpm of C^{14}, both about 90% resistant to RNAase. For the specific dilution test, 0.05 ml of the solution (acid-insoluble radioactivity, 326 cpm P^{32} and 142 cpm C^{14}, of which 287 and 132 cpm, respectively, was RNAase-resistant) and MS2 RNA or ribosomal RNA, as indicated, in 0.04 ml of 2.5 × SSC were mixed, heated, and processed as described under *Methods* for the annealing assays. Samples processed in an identical fashion but without addition of MS2 or ribosomal RNA had 260 cpm of P^{32} and 114 cpm of C^{14} of acid-insoluble radioactivity after RNAase digestion. The values of these controls were taken as 100% for the dilution test. All determinations were carried out in duplicate. Curves *1* and *2*, addition of MS2 RNA; curves *3* and *4*, addition of *E. coli* ribosomal RNA. Curves *1* and *3* (o-o-o), P^{32} label; curves *2* and *4* (x-x-x), C^{14} label.

appropriate plotting, yield straight lines fitting the experimental data. The amount of radioactive "plus" and "minus" strands present may then be obtained by extrapolation. The quantitative treatment of the annealing assays will be described in detail elsewhere. In this way 86 per cent or more of the C^{14}-labeled synthetase product was shown to be virus-specific, and 90 per cent of this to consist of parental-type, "plus" MS2 RNA strands. Astier-Manifacier and Cornuet[12] have independently used similar assays to demonstrate the formation of turnip yellow mosaic virus RNA in extracts of infected cabbage leaves.

Since the bulk of the RNA produced by RNA synthetase is MS2 RNA of the parental type, this enzyme must be concerned with the second step of RNA replication, i.e., the formation of progeny RNA. The enzyme appears to use endogenous, probably tightly bound replicative form as a template for the synthesis of viral RNA through base pairing with the "minus" strands of the duplex.[7] The fact that some of the newly synthesized "plus" strands are present in the duplex suggests a semiconservative mechanism whereby some of the growing "plus" RNA strands displace their counterparts from the double-stranded template. This is in line with the results of *in vivo* experiments both with MS2[6] and R17 phage,[13] as well as with turnip yellow mosaic[14] and encephalomyocarditis virus.[15]

There are indications that a phage-induced enzyme other than RNA synthetase is responsible for the first step of replication, namely, the conversion of single-stranded viral RNA to its double-stranded replicative form, by synthesizing complementary "minus" strands.[16, 17] Thus, both replication steps now appear to be amenable to study at the enzyme level.

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[1] Abbreviations: RNA and DNA, ribonucleic acid and deoxyribonucleic acid; ATP, GTP, UTP, and CTP, the 5'-triphosphates of adenosine, guanosine, uridine, and cytidine, respectively; RNAase A, pancreatic ribonuclease A; DNAase, pancreatic deoxyribonuclease; EDTA, ethylenediamine tetraacetic acid (the trisodium salt was used throughout); Tris, tris(hydroxymethyl)-aminomethane; SSC, 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7; dpm, disintegrations per minute; cpm, counts per minute; A_{260}, absorbance at 260 mμ, 1.0-cm light path; "plus" strands are defined as viral RNA strands of the parental type as opposed to "minus" strands which have the complementary base sequence.

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*DNA ASSOCIATED WITH TOBACCO CHLOROPLASTS**

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The existence of extranuclear genetic factors in a wide variety of organisms is well established.[1–3] Using equilibrium density gradient centrifugation, Chun, Vaughan, and Rich demonstrated that a difference in buoyant density exists between the DNA associated with chloroplast preparations and the DNA obtained from nuclear fractions of plant and algal cells.[4] Other investigators have also found DNA associated with the chloroplasts of *Chlamydomonas*,[5] *Euglena*,[6] and Swiss chard.[7] Lyttleton and Petersen,[8] however, found only one species of DNA in tobacco and suggested that if DNA was present in tobacco chloroplasts, its base composition was similar to that of tobacco nuclear DNA. The experiments reported below