In vitro Synthesis of a Segment of Bromegrass Mosaic Virus Ribonucleic Acid

(Accepted 20 February 1970)

Extracts of barley leaves infected with bromegrass mosaic virus (BrMV) incorporated radioactivity into RNA when incubated with [3H]UTP in the presence of actinomycin D (Semal & Kummert, 1970). The radioactive product thus obtained was partly resistant to pancreatic RNase in \( \times 2: \) SSC (0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0: SSC) but was entirely hydrolysed by this enzyme in \( \times 0.05: \) SSC (Semal, 1970). A sequential synthesis of double- and single-stranded RNA was obtained under certain experimental conditions (Semal & Kummert, 1971). The present results identify the radioactive product associated with duplex RNA as a segment of BrMV-RNA.

Cell-free extracts of the second leaf of barley seedlings, whose first leaf had been inoculated 3 days earlier with BrMV, were used to prepare the crude virus-induced RNA polymerase fraction. This fraction was incubated for 3½ min. with tritiated UTP (1 to 2 Ci/mM - The Radiochemical Centre, Amersham, Buckinghamshire, England), 10⁻⁵ M-EDTA, 20 μg./ml. of actinomycin D and the necessary ingredients for RNA synthesis, as previously described (Semal & Kummert, 1971).

The effect of various combinations of nucleotides in the incubation mixture on the level of labelling of RNA is shown in Fig. 1. Compared to that obtained with the complete mixture, radioactivity incorporation was inhibited by 70 to 80 % when CTP or GTP was omitted, and was inhibited by 95 % when ATP was omitted. Thus, labelling of RNA depended on the presence of all four ribonucleotides.

To see whether the RNase-resistant radioactive product was double-stranded RNA, annealing experiments were done: when the crude RNA product of the reaction was heated (10 min. at 100° in \( \times 0.1 \) SSC) and immediately cooled in ice, it was made almost entirely acid-soluble (2 to 5 % residual radioactivity) by RNase in \( \times 2: \) SSC (5 μg./ml. of pancreatic RNase A from Sigma Chemicals Co., incubated for 30 min. at 37°). A similar amount of enzyme-resistant radioactivity was obtained when heated material was adjusted to \( \times 2: \) SSC and kept for 16 hr at 80°. One reason for the apparent lack of self-reannealing could be interference by unlabelled single-stranded RNA, so attempts were made to purify the RNase-resistant material before heating.

In one set of experiments the crude RNA product was adjusted to 2 M-LiCl and kept overnight at 4°. After centrifuging for 10 min. at 10,000 g to eliminate the bulk of single-stranded RNA the supernatant was mixed with two volumes of ethanol and the insoluble fraction was used for the annealing experiments. But this gave little increase in RNase resistance after the heat-denatured RNA had been kept for 16 hr at 80°.

Subsequently, a more elaborate procedure was used to purify the RNase-resistant RNA product. The crude RNA product was dissolved in \( \times 2: \) SSC and was incubated successively for 30 min. at 37° with RNase (50 μg./ml.) and Pronase (500 μg./ml., Calbiochem, Inc., Los Angeles, California, U.S.A.) (Bové, 1967). The material was then deproteinized by emulsifying twice with phenol, and the water phase was mixed with two vol. ethanol. The precipitate thus obtained was dissolved in \( \times 0.1: \) SSC and was filtered through a column of Sephadex G-200 (16 cm. long, 1.5 cm. diameter) equilibrated with \( \times 0.1: \) SSC. The radioactive
fractions associated with the elution front were pooled. Samples (0.1 ml.) were heated for 10 min. at 100°, adjusted to ×2:SSC (final volume, 0.2 ml.) and incubated for 16 hr at 80°. Temperature was then slowly lowered; the samples were adjusted to 2 ml. of ×2:SSC and incubated with RNase. Under those conditions material that contained 2 to 6% RNase-resistant radioactive product immediately after heating, yielded 56 to 93% of RNase-resistant material. Such levels of self-reannealing allowed us to study the interference of different single-stranded RNAs with the reannealing process. Samples (0.1 ml.) of the solution obtained after filtration on Sephadex G-200 were mixed with 0.05 ml of ×6:SSC and 0.05 ml.

![Graph](image)

Fig. 1. Effect of nucleotides on the labelling of RNA synthesized by the crude polymerase preparation, upon incubation with [3H]UTP in the presence of actinomycin D. Results expressed as acid-insoluble counts × 10^{-8}/min./assay. • •, Addition of ATP+CTP+GTP; ○ ○, addition of ATP+CTP; △ △, addition of ATP+GTP; □ □, addition of CTP+GTP.

of different RNAs in ×2:SSC. Four types of RNA were used: those from BrMV, tobacco mosaic virus (TMV) broad bean mottle virus (BBMV) and ribosomal RNA (wheat germ), purchased from Calbiochem Inc. the viruses were prepared by clarification of extracts of infected leaves with chloroform, followed by ultracentrifugation. Virus suspensions were deproteinized with phenol, and RNA was precipitated with ethanol. RNA concentrations were estimated by ultraviolet absorbence. In one experiment the water phase obtained after phenol treatment of BrMV was extracted three times with ether and then freed from ether by bubbling nitrogen; the results were similar to those of ethanol-precipitated RNA. Fig. 2 shows the results of interference experiments. They show clearly that self-reannealing of the heat-dissociated strands is completely inhibited by low concentrations of BrMV-RNA but is unaffected by TMV-RNA, BBMV-RNA or ribosomal RNA. This result indicates that the RNA synthesized in vitro during a short pulse of [3H]UTP and the RNA of BrMV particles contains one and the same sequence, and this is presumably a segment of the ‘plus’ strand (Weissmann & Ochoa, 1967). Incomplete self-reannealing in some experiments may be caused by low concentration of RNA duplex. There was no indication that ‘minus’ strands of BrMV-RNA are synthesized in the system we have used.
Astier-Manifacier & Cornuet (1965) and Bové (1967) have reported heat dissociation and reannealing of RNA synthesized *in vitro* by leaf preparations from cabbage infected with turnip yellow mosaic virus, but in similar experiments with cucumber mosaic virus specific self-reannealing of double-stranded RNA synthesized *in vitro* was not established (May, Gilligand & Symons, 1970), possibly because of incomplete purification of the RNA duplex.

This work was supported in part (J.S.) by the Fonds National de la Recherche Scientifique, Brussels, and in part (J.K.) by the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture Brussels. We are grateful to Dr A. Burny and Dr W. Fiers for helpful suggestions, and to Dr M. Chodkiewicz, Merck, Sharp and Dohme Research Laboratories, for a sample of actinomycin D. We express our sincere appreciation to Misses Anne-Marie Pollart and Paulette Janssens for able technical assistance, and to Messrs E. François and A. Riga, Station de Physique et Chimie agricoles, Gembloux, for help with radioactivity measurements.

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(Received 19 January 1971)