TURNIP YELLOW MOSAIC VIRUS-RNA SYNTHESIS IN VITRO : EVIDENCE FOR NATIVE DOUBLE-STRANDED RNA.

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78 - VERSAILLES

Received June 26, 1968

A cell-free, particulate fraction isolated from chinese cabbage (Brassica chinensis) infected with turnip yellow mosaic virus (TYMV) is able to synthetize RNA of the viral type ("plus" strand) in the presence of the four nucleoside 5'-triphosphates and Mg^{++} (Bové, 1967a). The template for this asymmetric RNA synthesis is a "minus" strand formed in vivo, bound to the cell-free preparation and isolated together with it. (Bové, 1966 and 1967 b). After deproteinization by phenol, the product of this replicase reaction is associated with a minus strand in a double-stranded RNA structure (Bové, 1967b).

Double-stranded RNA (replicative form, replicative intermediate ; Franklin, 1966) has generally been implicated in the replication of viral RNA. However it has recently been suggested that these double-stranded RNA structures may arise as artifacts following treatment by phenol or sodium dodecylsulfate (Feix et al., 1968). The replicating complex, in its native state, would be predominantly single-stranded, the template ("minus" strand) and the nascent product ("plus" strand) not being extensively hydrogen-bonded to each other (Feix et al., 1968).

In the experiments reported here, we have reinvestigated the RNase-resistance of the product of the TYMV - RNA replicase reaction and we present evidence that it occurs in a double-stranded form at the end of the in vitro synthesis even without deproteinization by phenol.

METHODS

The isolation of the cell-free preparation, 100 P_2 10,000, from TYMV-infected chinese cabbage leaves, the composition of the reaction mixture and conditions for DNA-independent RNA synthesis have been described previously (Bové et al., 1965 ; Bové, 1967b).

RNA for sucrose gradient centrifugation was purified by two phenol-extractions, chromatography on Sephadex - G 50 and by precipitation with ethanol. The RNA was finally dissolved in 1 X SSC buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.4).

The sucrose solutions for the 5 % - 20 % linear gradients were prepared with 1 X SSC buffer, in the SW 25.I rotor of the Spinco centrifuge, model L. The tubes were centrifuged for 15 hours at 24,000 rpm and 5° C. The bottom of each tube was punctured and the contents were allowed to flow through a capillary tubing connected to a flow cell (2 mm light path) of a Beckman DK2 spectrophotometer permitting the recording of the optical density at 260 mμ. After the flow cell, the tubing was led under the window of a Geiger-Müller detector in order to record radioactivity. Fractions of 1.2 ml were collected and used for the determination of trichloracetic acid (TCA) - precipitable radioactivity before and after pancreatic RNase treatment.

RESULTS AND DISCUSSION

The cell-free preparation, 100 P_2 10,000, isolated from TYMV-infected leaves contains both replicase- and RNA-polymerase activities. The synthesis of RNA by the DNA-dependent RNA-polymerase can be prevented by the addition of actinomycin D in the reaction mixture. As illustrated by fig. 1, the product obtained in the presence of actinomycin D (replicase reaction) represents approximatively 50 % of the total RNA obtained in the absence of antibiotic. In the experiment of fig. 1, after a 30 min reaction at 37° C, the product of the replicase-reaction synthetized in the presence of actinomycin D was tested for resistance to pancreatic RNase in 0.1 X SSC or 1.0 X SSC buffer. Fig. 1 shows that the product is RNase-resistant at high ionic strength (1.0 X SSC) as would be expected from a double-stranded RNA, but that at low ionic strength (0.1 X SSC), where double-stranded RNA is known to be highly RNase-sensitive (Billeter et al., 1966), only 35 % are hydrolyzed by the RNase. Essentially the same results are obtained when DNA-independent RNA-synthesis is carried out at 10° C instead of 37° C.

If, at the end of a 30 min incubation carried out in the presence of actinomycin D at 37° C or at 15° C, the reaction mixture is diluted from 0.4 ml to 8.0 ml with 0.1 X SSC or 1.0 X SSC buffer, and centrifuged at 30,000 g for 15 min, one finds that 80 to 90 % of the replicase-product are bound to the pellet. The small fraction that remains in the supernatant is RNase-sensitive at low ionic strength and RNase resistant at high ionic strength ; under condition of high ionic strength where it is resistant to pancreatic RNase, it is hydrolyzed by RNase III, an enzyme specific for double-stranded RNA (Robertson et al., 1967). Thus the product in the supernatant is double-stranded RNA.

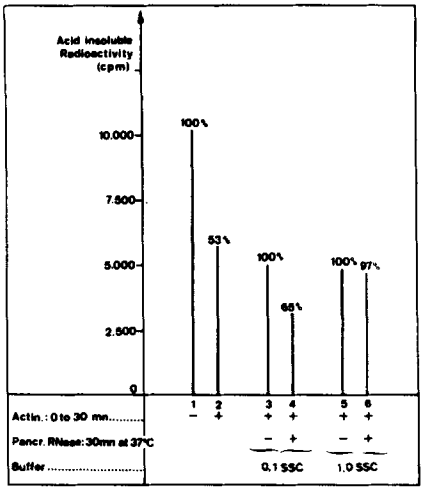

Figure 1

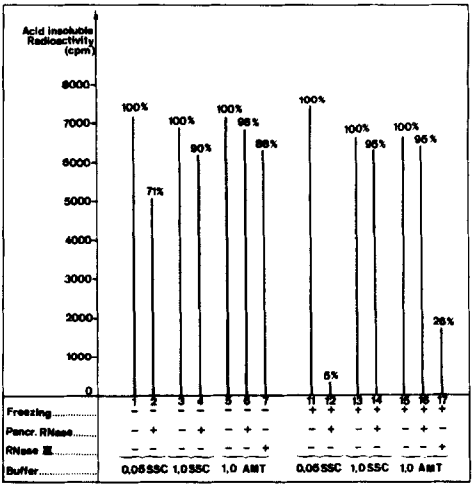

Figure 2

Figure 1 - RNase-resistance of the replicase-product (E 259). Each reaction mixture contained in a total volume of 0.40 ml, in µmoles : Tris-HCl, pH 9.0 : 30 ; KCl : 2 ; $MgCl_2$: 2 ; ATP, CTP and UTP : 1 ; GTP-^{32}P : 0.06 (97 µCi/µmole) ; Phosphoenolpyruvate : 5. Pyruvate-kinase : 20 µg. Actinomycin D, where indicated : 50 µg. After 30 min at 37° C, reactions 1 and 2 were stopped with 3 ml of 15 % TCA ; reaction mixtures 3, 4, 5 and 6 were diluted to 4.0 ml with 0.1 or 1.0 X SSC buffer as indicated, and RNase was added to tubes 4 and 6 to the final concentration of 0.8 mg/ml. After 30 min at 37° C, 0.4 ml of 100 % TCA was added to tubes 3, 4, 5 and 6. Radioactivity of the acid-insoluble precipitate from each reaction mixture was determined as described previously (Bové et al., 1965).

Figure 2 - Effect of freezing and thawing of the pellets on the RNase-resistance of the replicase-product (E 280).
The composition of the reaction mixture was the same as for fig. 1. Actinomycin D : 50 µg per 0.4 ml of reaction mixture. The replicase-reaction was run at 15° C for 60 min. The RNase-test (100 µg RNase per ml) was also done at 15° C for 60 min.

As shown by figure 2 (1 to 7), the replicase product in the pellets is resistant to pancreatic RNase to the extent of 90 % or more in 1 X SSC or 1 X AMT buffer (1 X AMT is 0.15 M NH_4Cl, 0,015 M Mg-acetate, 0,03 M Tris-HCl, pH 7.4 at 25° C), but in 0,05 SSC only 30 % of the product become sensitive to pancreatic RNase. Also the resistance to RNase III is high (88 %). These results suggest that a major part of the replicase product bound to the pellet is somehow protected against the action of pancreatic RNase and RNase III. To render the product accessible to the

ribonucleases, we have looked for a treatment that would alter the structure of the particles (chloroplasts, nucleoli) in the pellet without favoring the formation of hydrogen bonds between complementary single-stranded RNA eventually present.

Freezing (2 min at - 70° C) followed by thawing (3 min at + 15° C) is a treatment under which single, complementary RNA strands resulting from heat-melted TYMV double-stranded RNA (5 min at 120° C in 1 X SSC), do not reanneal. When the pellets, resuspended in 0.5 ml of 0.05 SSC, are twice submitted to this treatment, the replicase product shows great RNase-sensitivity at low ionic strength (tube n° 12) and almost total RNase-resistance at high ionic strength (tube n° 14). Also 75 % of the product are now sensitive to RNase III, as against 12 % (tube n° 7), in the absence of the freeze and thaw treatment.

These experiments show that the product of the replicase reaction is part of a double-stranded structure at the end of the in vitro synthesis prior to any phenol or SDS treatment.

Hydrogen bonding between the minus strand and the nascent product to form double-stranded RNA could possibly occur as an artifact during in vitro synthesis, if the temperature at which the replicase reaction was run, namely 37° C, was too high. Therefore all experiments have been carried out not only at 30 or 37° C but also at 10 or 15° C. The results were entirely the same at 10° C than at 37° C. Thus the occurence of the replicase product in a double-stranded structure at the end of the incubation does not seem to be the result of an artifact due to a too high a temperature of reaction.

The results of figures 1 and 2 give information only about the labelled product of the in vitro synthesis itself ; in other words, they show that only the product formed and labelled in the course of the reaction is hydrogen bonded to the corresponding complementary regions of the minus strand ; those that have not been used as template during the reaction, could be single-stranded. If so, treatment of the particles of the pellet with pancreatic RNase in 1 X SSC buffer, after freezing and thawing, should result in the hydrolysis of the single-stranded regions but not of the double-stranded parts containing the labelled product of the reaction. Thus, after phenol deproteinization, the labelled double-stranded RNA from the RNase-treated pellet should sediment in the 5 to 20 % sucrose gradient with a much smaller sedimentation coefficient (S) than the double-stranded RNA from the pellets not submitted to the RNase treatment, and which is known, from previous experiments, to have a S value of 15 to 17.

In the experiments of fig. 3 and 4, the reaction mixture from

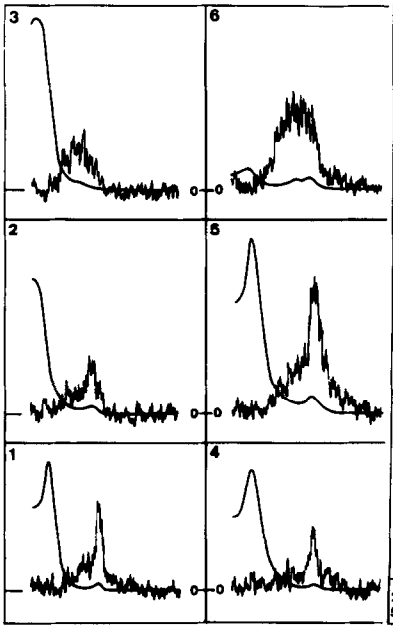

Figure 3

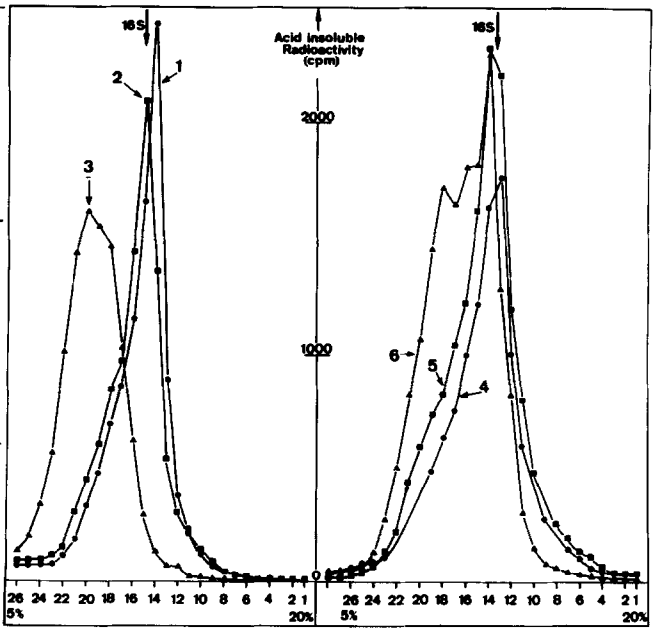

Figure 4

Figure 3 - Recordings of absorbance at 260 mμ (——) and of radioactivity (- - - -) of the RNA after sucrose density gradient centrifugation. (E 285). 1, 2 and 3 : RNA from preparation "I" (no RNase treatment of the replicase product in the pellet). Three identical aliquots of the RNA solution in 1 X SSC buffer were placed at 30° C, one without RNase (1), one with 0.5 μg RNase/ml (2), and one with 50 μg RNase/ml (3). After a 30 min incubation, the solutions (0.50 ml) were placed immediately on top of the sucrose gradients, without removing the RNase.

4 : Same as 1.

5 : RNA from preparation "II" (pellet treated with RNase : 0.5 μg/ml)

6 : RNA from preparation "III" (pellet treated with RNase : 50.0 μg/ml)

Gradients 1, 2 and 3 were centrifuged together ; so were gradients 4, 5 and 6.

Figure 4 - TCA-precipitable radioactivity from the sucrose gradients 1, 2, 3, 4, 5 and 6 of figure 3. (E 285).
1.2 ml fractions were collected with a siphon ; 0.6 ml of each fraction was used directly for TCA precipitation ; the other half was incubated in 1 X SSC buffer for 30 min at 30° C with RNase (50 μg/ml) before TCA precipitation. Practically the same figures were obtained in both cases and thus the RNase-resistant radioactivity is the same as that indicated on the figure.

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a large-scale synthesis (30 min at 30° C) was diluted 10 times with 0.05 X SSC buffer and centrifuged for 15 min at 30,000 g. The pellets were

resuspended in 0.05 X SSC buffer and submitted to the freeze- and- thaw treatment. Following this treatment, the product of the replicase reaction in the pellet was sensitive to RNase in 0.05 X SSC but resistant in 1.0 X SSC as determined on separate controls. Concentrated SSC buffer was added to the resuspended pellets to the final concentration of 1 X SSC. One third of the suspension was placed at 30° C for 30 min without RNase (preparation I), the second and third parts were also placed at 30° C for 30 min but in the presence of RNase at the final concentration of 0.5 µg/ml (preparation II) or 50 µg/ml (preparation III) respectively. RNA was extracted and analyzed by 5 - 20 % sucrose density gradient centrifugation, as described in "Methods".

As illustrated by fig. 3 and 4, the product of the replicase reaction, obtained after phenol deproteinization, sediments in the 15 - 17 S region (gradients 1 or 4). Treatment of this isolated RNA with RNase at a low concentration (0.5 µg/ml) shifts the radioactivity peak only slightly toward the lower S region (gradient 2) ; with a higher RNase concentration a single peak in the 10 S region is obtained (gradient 3). No radioactivity appears on the surface of the gradient, indicating that the 15 - 17 material is totally double-stranded.

As further shown by fig. 3 and 4, no difference in sedimentation behavior could be seen between the RNA from the control preparation (gradient 1 or 4) and the RNA from the pellets treated with RNase at a low concentration (gradient 5). The treatment of the pellets with RNase at a high concentration has modified the sedimentation behavior of the replicase product (gradient 6) ; part of it now sediments with a lower velocity in a way similar to that of the 15 - 17 S RNA after treatment with RNase at a high concentration (gradient 3).

The experiments of fig. 3 and 4 thus show that after freezing and thawing of the pellets, a treatment which renders the replicase product accessible to ribonuclease, without favoring reannealing, the addition of RNase to the pellets does not yield, after phenol extraction, fragments of double-stranded RNA smaller than those obtained by RNase treatment of the isolated 15 - 17 S double-stranded RNA. Therefore the regions of double-strandedness must be as high in the RNA in the pellet before phenol extraction than in the 15 - 17 S double-stranded RNA obtained after phenol extraction. It seems thus unlikely that hydrogen-bonding occurs only along short regions of the minus strand such as those where nascent product is being made. On the contrary, these experiments are more in agreement with the idea that the minus strand is predominantly in a double-stranded form at the end of the incubation. They do not however

exclude the possibility that single-stranded regions along the minus strand may exist before incubation.

Acknowledgements

We wish to thank Dr. C. Weissmann for many helpful discussions and for advice.

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