IDENTIFICATION AND CHARACTERIZATION OF AN RNA REPLICASE FROM TMV-INFECTED TOBACCO LEAVES

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SUMMARY

An RNA-dependent RNA polymerase, replicase, has been isolated from systemically TMV-infected tobacco leaves. Gelfiltration on Sephadex G-100 of the supernatant, after high speed centrifugation of the sap, shows a new peak which is only to be found in infected material. Replicase activity is eluted close to that new peak as a molecule with a molecular weight which can be about 70,000. The enzyme needs a TMV-RNA-primer for full activity.

The formation of infectious ribonucleic acid during the multiplication of tobacco mosaic virus (TMV) has been described in vivo (Engler and Schramm, 1960). In vitro, the synthesis of RNA in crude extracts of tobacco plants is difficult to observe because of the relatively high content of nucleases released during the extraction of plant enzymes. Some partly purified preparations with regard to replicase (RNA-dependent RNA polymerase) activity in this system also have failed (Gilliland et al., 1968; Semal et al., 1968). Evidently an effective separation of the nucleases from the replicase or an inhibition of these nucleases is necessary.

MATERIALS AND METHODS

Virus. The flavum strain of TMV was used in most of the experiments, and a wild type strain was also used in some experiments. The virus was purified essentially as described by Steere (1963).

Host plants. Nicotiana tabacum L. var. "Samsun" grown in soil in 10 cm pots were used as host plants. During the experiments the plants were kept in a growth chamber with illumination from fluorescent lamps and a day length of 16 hrs. The light intensity was 6,000 lux, the day temperature 25° and the night temperature 20°. The first leaf which displayed a pointed tip,

normally number 7 including the cotyledons, was dusted with carborundum and inoculated when the plants had 11-13 visible leaves. (Sap from infected leaves was used as inoculum.) The inoculated leaf was carefully rinsed with tap water. With this procedure under growth chamber conditions the leaf, which had a length of 20-35 mm at the time of inoculation, and usually number 9-11, showed vein clearing on the major part of the lamina after 4-5 days (Zech, 1952). This leaf was used in the various experiments.

Reagents: ^{14}C-labeled uridine triphosphate was from Amersham, England and was used at the specific activity 56 mCi/mM.
Unlabeled riboside triphosphates, ribosomal yeast RNA, pancreatic deoxyribonuclease (DNAase, E.C. 3.1.4.5) and pancreatic ribonuclease (RNAase, E.C. 2.7.7.16) were purchased from Sigma Chemical Company.
Phosphoenolpyruvate (PEP) and the corresponding pyruvate kinase (PEP-kinase, E.C. 2.7.1.40) were from C.F. Boehringer & Soehne, Mannheim, Germany. TMV-RNA was prepared by extraction with phenol in the presence of bentonite according to Fraenkel-Conrat et al. (1961).
Turnip yellow mosaic virus (TYMV) RNA and Holmes' Ribgrass virus (HRG) RNA were kindly provided by Dr. W. Mundry, Max Planck Institut für Biologie, Abteilung Melchers, Tübingen, Germany.

Preparation of the enzyme
About 17 g of frozen infected leaves were first ground in a prechilled mortar and then pressed in the presence of 0.05 M sodium cacodylate buffer pH 7.2, 0.1 M ammonium sulfate and 0.5 mM glutathione. The extract was centrifuged at 48,000 g for 45 min at 4° in a Sorvall centrifug (RC 2-B).

Gelfiltration of the supernatant was carried out on a Sephadex G-100 column in 0.05 M sodium cacodylate buffer, pH 7.2 at 4°.

Assay of replicase activity. The standard reaction mixture of 0.25 ml contained 50 mM sodium cacodylate pH 7.2, 12.8 mM $MgCl_2$, 0.8 mM $MnCl_2$, 1.1 mM KCl, 42.0 mM NH_4Cl, 0.8 mM ATP, 0.8 mM CTP, 0.8 mM GTP, 0.8 mM ^{14}C-labeled UTP ($5x10^6$ cpm/µmole), 18.5 µg/ml of DNase, 0.2 mM glutathione, 20 µg/ml of pyruvate kinase and 5 mM of phosphoenolpyruvate, 10 µg (and later 5 µg) of the nucleic acid was used as template. Enzymes were assayed at levels of 50-400 µg protein per sample. Incubations were carried out at 35° for 30 min and terminated by placing the reaction mixture in an ice bath and with addition of 0.5 ml of 10% trichloracetic acid (TCA) containing 0.9% Na pyrophosphate.

The precipitate was washed onto a glass fiber filter (Whatman GF/C) and washed five times with 10 ml of cold TCA-solution as described above. The glass fiber filter was then dried and counted in a Packard liquid scintillation spectrometer (Model 3315).

Assay of Ribonuclease activity was performed according to Reddi (1967).

RESULTS

In the gelfiltration on Sephadex G-100 (Fig. 1) the small peak, or the shoulder between the first and the last peaks is only found in infected material. From gelfiltrations, with reference substances on the same column, the replicase elutes as a molecule corresponding to bovine serum albumin (mol. wt. 69,000). Sometimes replicase activity can also be noticed after the shoulder. Reference substances with molecular weights about 20,000 elutes within this region.
(None of the fractions with replicase activity had concentrations high enough for analytical ultracentrifugation.) Ribonuclease activity was demonstrated almost symmetrically under the shoulder.
During the test, ribosid triphosphate generating system (PEP and PEP-

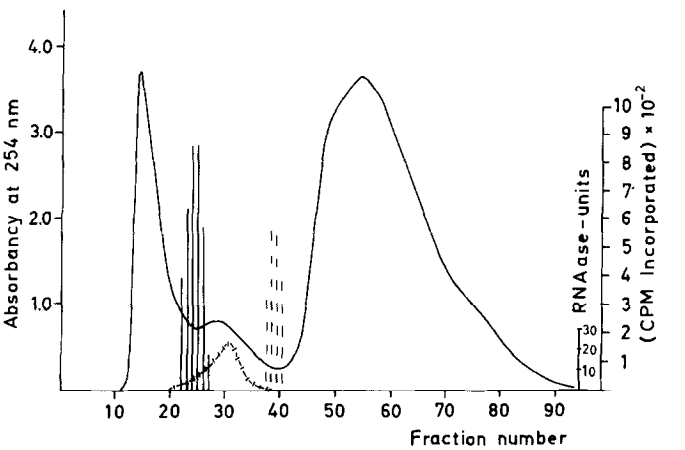

Fig. 1 Gelfiltration of 5 ml of the supernatant from the preparation of the enzyme on Sephadex G-100, column size, 3.2 cm x 21.3 cm, total volume 166 ml. Buffer 0.05 M Na-cacodylate (pH 7.2); temperature, 4°; elution rate, 29.2 ml/h; fraction volume 3 ml. The replicase activity, (CPM Incorporated) x 10^{-2}, is denoted in the figure by vertical lines where the activity generally appears and by lines of short dashes where the activity is sometimes also noticed. Activity of RNAase is denoted by -/-/-.
(A unit of RNAase activity is the amount of the enzyme needed to cause an increase in the absorbancy of 0.005 at 260 nm).

kinase) was included to avoid contributions by RNA-phosphorylase to the observed incorporation and the action of ATPase. Sometimes an excess of ATP has been used instead and has given the same result. DNAase was added to eliminate DNA-dependent synthesis.

Some of the general characteristics of the enzyme is shown in Table 1. The replicase needs a specific template for full activity.

Table 1

Template Specificity of Replicase

Template (all at 10 μg/0.25 ml)	Activity in per cent of the TMV-RNA value
TMV-RNA	100
TYMV-RNA	47
HRG-RNA	23
Yeast Ribosomal RNA	0
0	0

Table 2

Triphosphate Dependence of Enzyme

Assay mixture	Template	Activity in per cent of the "complete"-value
Complete	TMV-RNA	100
- CTP	"	41
- GTP	"	24
- ATP	"	27
Complete	0	0

Table 2. shows the dependence of nucleoside triphosphates.
Figure 2. shows the dependence of the amount af added template. Between 5 and 10 µg there is a plateau, so in the following experiments the lower amount has been used.
The reaction was also executed in the presence of pancreatic ribonuclease (40 µg) added to the assay. The activity then dropped down to 7%. However, if the addition of RNAase was made after the incubation had proceeded for 20 min the activity was about 25%.

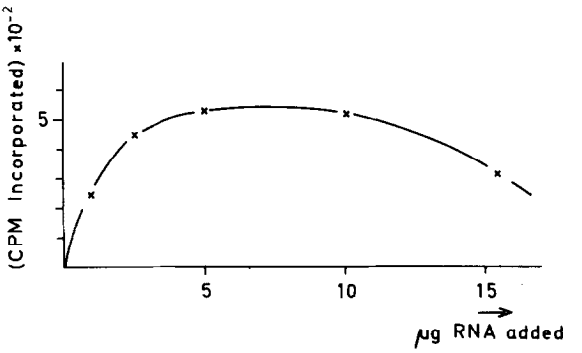

Fig. 2 Variation of the replicase activity, (CPM Incorporated) x 10^{-2}, with the amount of added TMV-RNA-primer.

The higher value in the second case may depend upon a formation of some double stranded RNA.

DISCUSSION

After the enzyme purification described above, the replicase needs an RNA template for activity. The specificity is not absolute. Because DNAase is always present in the assay the activity can be distinguished from the DNA-primed polymerase. The use of riboside triphosphates distinguishes this enzyme from RNA-phosphorylase. To obtain full activity all four of the nucleoside triphosphates are necessary in the assay mixture.
Because of the requirement of a template for the initiation of activity and the sensitivity to ribonucleases, the product must consist of nucleic acids.
The main problem evidently is to eliminate nucleases since good inhibitors against them are yet to be found. Ion exchange chromatography seems to be the most suitable possibility in spite of the difficulties with plant proteins on charged materials.

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