ISOLATION OF TURNIP YELLOW MOSAIC VIRUS RNA REPLICASE AND ASYMMETRICAL SYNTHESIS OF POLYNUCLEOTIDES IDENTICAL TO TYMV-RNA

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The current concept on viral RNA replication is that intermediary double-stranded RNA (Montagnier and Sanders, 1963) and a newly formed replicase coded by the virus genome are involved in the process. The replicase primed by double-stranded RNA is thought to synthesize asymmetrically the infectious single-stranded RNA.(Weissmann et al,1964).

We are reporting here on the isolation of a plant virus RNA replicase from infected Chinese cabbage capable of synthesizing in vitro free single-stranded polynucleotides identical to Turnip Yellow Mosaic Virus-RNA (TYMV-RNA).

EXPERIMENTAL

1) Isolation of replicase. 11 days after inoculation with TYMV, 100g of infected Chinese Cabbage leaves were frozen and ground in a blender, with 200ml of 0.25 M Sucrose containing 0.01 M Tris-Citric Acid buffer pH 7.5, and 0.005 M $MgCl_2$. The ground material was centrifuged for 15 min at 15,000g Supernatant was made 0.007 M with EDTA. After 5 min at 0°C, the mixture was centrifuged for 15 min at 15,000 g. The pellet was dissolved two times in 30 ml Tris buffer, 0.01 M $MgCl_2$ and all insoluble material was eliminated by centrifugation. The supernatant was run through a column of DEAE-cellulose (2g) and washed with 50 ml of a solution containing Tris-Citric acid buffer pH 7.5, 0.005 M $MgCl_2$, 0.05 M NaCl. A similar solution which was 0.3 M in NaCl was then used to elute the enzyme. The protein was con-

centrated by a 1/1 addition of saturated ammonium sulfate and this precipitate was suspended in 1 or 2 ml of 0.01 M Tris-HCl buffer pH 7.5 containing 0.0I25 M $MgCl_2$ and 0.005 M Mn Cl_2. All operations were performed at 0° - 4°C.

2) Synthesis of TYMV complementary RNA. DNA-RNA transcriptase was purified from Micrococcus lysodeikticus by a method similar to that described by Nakamoto et al (1963). The 1.25 ml of reaction mixture contained the following : Tris-HCl pH 7.5 : 125 μM ; Mn Cl_2, 3.1 μM ; ATP, CTP, GTP, UTP, 3 μM each. In addition, it contained DNase, 100 μg ; TYMV-RNA, 250 μg ; enzyme 2 mg. Incubation was carried out at 30°C for 30 min. Next, 30 μg/ml of ribonuclease was added to the mixture at 0.6 M NaCl, 0.06 M Sodium citrate (4 SSC), and incubated 25 min at 27°C. The ribonuclease-resistant double-stranded RNA was extracted with phenol and then precipitated at pH 2.5 and dissolved in 2 SSC.

3) Denaturation and reanneling. Denaturation was performed at 108°C in 2 SSC for 6 min. For reanneling, the temperature was then adjusted to 85°C, for 2 hours. The ribonuclease resistant product was obtained as described above.

RESULTS

In order to show clearly the TYMV-RNA synthesis by incorporation of nucleotides into the acid-insoluble material, it was necessary to eliminate confusing parasitic reactions from the system. For that purpose the incubation was carried out with desoxyribonuclease to reduce DNA-RNA transcriptase. The enzymatic extract was preincubated with GTP to minimize incorporation of GTP.

The comparison between similar fractions isolated from healthy and infected plants (Table I) shows that :

1. The level of incorporation in the extracts from infected plants is

ten times that of healthy ones.

2. The C/G ratio is very characteristic of TYMV-RNA. During the infection, the C/G ratio (percent incorporation) increases to nearly the value given by virus analysis (Fox et al, 1964).

From numerous experiments we have concluded that TYMV-RNA primer was unsuccessful in increasing incorporation, but in some cases extracts from infected plants were stimulated by a double-stranded TYMV-RNA primer (see Methods).

TABLE I

	Healthy plants	%	7 days infected plants	%	11 days infected plants	%	Virus analysis
A	3.4	21	52.5	23	32.7	21	20
C	4.0	25	64.9	29	62.7	40	39
G	6.7	42	64.8	29	41.6	27	19
U	1.7	10	36.7	16	17.0	11	22

AMP, CMP, GMP, UMP in μμM incorporated. The final concentration of the incubation medium was : Tris-HCl buffer pH 8.5, 0.08M ; Sodium succinate, 0.08M ; $MgCl_2$, 0.005M ; Mn Cl_2, 0.002M. Each sample, 0.25 ml, contains: ATP 40, CTP 44, GTP 41, UTP 45 mμM, and 0.1 μc of the ^{14}C-labelled nucleotide; DNase 1μg; enzymatic extract 200-1000 μg protein in 0.1 ml. Preincubation: 30 min at 10°C. Incubation: 25 min at 30°C.

The above results suggest a similarity between the product synthesized in vitro and TYMV-RNA. To proove this point, hybridization and displacement by similar polynucleotides was necessary.

The Weissmann hybridization and displacement method is very convenient for double-stranded polynucleotides. Displacement of the radioactivity indicates that one strand is identical with the displacer. But we must expect the radioactive product to contain double-stranded and also free single-stranded polynucleotides. Therefore, it is necessary to make the free single-stranded RNA resistant to ribonuclease by hybridization with cold double-stranded viral RNA. (Robinson, et al 1964). Following the same line of thought, we may predict

that if the labelled product contains free single-stranded viral RNA, and if the enzymatic extract carries endogenous double-stranded cold primer, then denaturation and self reanneling must increase the ribonuclease-resistant radioactive product.

The results with enzyme A (Table 2) indicate that 1/8 of the total labelled product is resistant to ribonuclease. When this product is self-reanneled, the resistant radioactive product is increased. After reanneling with double-stranded cold viral RNA, the amount of ribonuclease-resistant product is nearly doubled. Consequently, the product of the replicase action must contain either free "plus" strands or "minus" strands of TYMV-RNA. Since the added cold single-stranded TYMV-RNA progressively displaced the radioactivity, it is clear that the

TABLE II

	Experimental			Results	
	D	R	RNase	A	B
Product	-	-	-	1356	
Product	-	-	+	168	
Product	+	+	+	215	68(c)
Product + cRNA	+	+	+	311	109(b)
Product + cRNA + TYMV-RNA(1μg)	+	+	+	277	81
Product + cRNA + TYMV-RNA(100μg)	+	+	+	245	57(a)
Product + cRNA + TMV-RNA (80μg)	+	+	+	312	109
Product	+	-	+	148	

Hybridization and displacement. The labelled replicase product was extracted three times with phenol, then precipitated by HCl at pH 2.5 and dissolved in 2 SSC. Aliquots (0.1 ml) were mixed with various cold polynucleotides in 2 SSC, and 50 μg of complementary TYMV-RNA (cRNA) was added as indicated. Then the mixtures were denaturated (column D), reanneled (column R), and treated with RNase, as indicated in the table. Radioactivity in cpm in the acid-insoluble product was isolated and counted.

strands are "plus". We may therefore conclude that a part of the synthesized polynucleotides is identical to TYMV-RNA. The results with enzyme B permit an estimate of the amount of free "plus" strands and "plus" strands inserted in double-stranded polynucleotides. (b-c) represents the free strands, (b-a) the total "plus" that could be displaced, so that the difference between these values represents the part of the "plus" strands that is combined with "minus" strands. The ratio (free strands/double strands) thus obtained is about 4. In the case of enzyme A, probably because of incomplete denaturation, we can be sure only that the strands are mostly "plus". These results suggest an asymmetrical process for the replicase system isolated from TYMV infected plants.

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