SYNTHESIS OF INFECTIOUS TOBACCO MOSAIC VIRUS RNA BY CELL FREE EXTRACTS OBTAINED FROM TMV INFECTED TOBACCO LEAVES

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Received May 23, 1962

When the lower epidermis of tobacco, or other leaves, is stripped away to expose the mesophyll, gentle rubbing of the mexophyll in the presence of a strong, dicarboxylic acid buffer will produce a sticky protoplasmic extract. This condition can be readily observed under a dissecting microscope when 0.5 M KOH-Maleate buffer, pH 6.9 is used as the homogenizing medium. The jelled protoplasmic extract arises because dicarboxylic acid buffers (also 0.5 M citrate) disrupt plant nuclei leaving the DNA in a highly polymerized, fibrous condition. Addition of crystalline DNAase will quickly destroy the jell, whereas crystalline RNAase has no effect on the jell. In the light of recent work on the extraction of DNA-RNA complexes from pea embryos (Huang, et al., 1960), and Neurospora (Schulman et al., 1962), and from the demonstration of the DNA dependent synthesis of RNA in preparations from liver nuclei (Weiss 1960) and bacteria (Hurwitz, et al., 1960), the question arose as to whether the jelled protoplasm from leaves infected with tobacco mosaic virus (TMV) would display similar properties which could be measured by the infectivity of TMV-RNA.

Our first experiments suggested that a crude, DNA-TMV-RNA complex could be isolated from leaves that had been infected with the common or U1 strain of TMV. Lower epidermis was stripped from leaves whose upper surface had been inoculated 14 days previously with 10^{-2} mg./ml. TMV. Drops of 0.5 M maleate buffer were applied to the mesophyll, the

cells gently disrupted, and the viscous, cell-free homogenate collected until about 1.2 ml. were obtained. The extract was kept at 0^{o} during preparation. The extract was centrifuged in an International Clinical Centrifuge for 10 minutes at top speed. The supernatant solution was withdrawn (1st wash), the precipitate resuspended in 0.5 ml. of maleate buffer, and the process repeated to produce 6 washes. Finally, the precipitate was suspended again in 0.5 M maleate, crystalline DNAase + $MgCl_2$ was added, the mixture allowed to incubate 1 hour at room temperature, centrifuged, and the 7th wash collected. Each of the washes was applied to Nicotiana glutinosa leaves to test for local lesion production by TMV. The results were: 1st wash, 394 lesions; 2, 70; 3, 41; 4, 43; 5, 29; 6, 14; 7 (after DNAase), 73. Apparently DNAase treatment released an infectious material from the crude extract. Subsequent experiments confirmed this finding, and also showed that the infectious material released into the supernatant was destroyed by RNAase. Thus, there appears to be a TMV-RNA in close association with the DNA contained in these crude extracts. The results were sufficiently suggestive to prompt us to test these extracts for possible synthesis of TMV-RNA.

Preparation of Extracts. We have tried a number of methods for preparing extracts, all of which have given evidence of an increase in infectivity when incubated for 1 hour with a combination of ATP, GTP, UTP, CTP and compared to extracts that received either no nucleotides, ATP alone, or three out of the four triphosphate nucleotides. Since our most recent method produces consistent results, we will confine our description to this method. Leaves of Nicotiana tabacum var. Turkish Samsun which were about 15 cm. long at the time of harvest and which had been directly inoculated with 10^{-2} mg/ml of U1 TMV 10 to 20 days previously were used.

Two, Gillett double-edged razor blades are separated by a teflon spacer ring and attached to the rotor arm of a Virtis Homogenizer. New

blades are used for each homogenate prepared. Midribs and lateral large veins of TMV infected tobacco leaves are dissected and discarded and the strips of lamina tissue retained. The razor blade assembly is inserted into a round, polyethylene jar, which in turn is surrounded by an ice bath. All operations are conducted in a cold room. 50 ml. of 0.5 M maleate buffer are added such that the razor blades are covered by liquid. With the variac set at 30, the strips of leaf tissue weighing 10 grams are subjected to the cutting action of the rotating razor blades. As soon as all of the tissue has been added, the cutting is stopped. The homogenization is inefficient, only about 10% of the tissue being rendered into a cell-free extract. Most of the tissue remains as small pieces of leaves which can be separated from the cell-free extract by filtration through two layers of cheesecloth. The filtrate, contained in two, 40 ml. tubes in swinging buckets, is spun for 15 minutes at top speed in the International Clinical Centrifuge. The supernatant solutions are discarded, and the small, heterogeneous, sticky, green and white pellets are gently resuspended in 10 ml. of 0.5 M maleate buffer, the suspensions combined into one tube, and re-centrifuged. The second supernatant is discarded, and the pellet re-suspended in 10 ml. of 0.5 M maleate buffer. Aliquots of the suspension are then distributed into separate, small centrifuge tubes, which are centrifuged and the maleate supernatants discarded. The pellets are then suspended into 0.5 to 1.2 ml. of the appropriate treatment media.

Incubation Media. Two media have been used. 1) Tris, pH 8, 100 uM/ml; $MgCl_2$, 5 uM; cysteine, 10 uM; KCl, 30 uM. 2) KH_2PO_4-K_2HPO_4, pH 7, 20 uM; $MgCl_2$, 2 uM; Mercaptoethanol, 0.3 uM. To these media were added triphosphate nucleotides such that the final concentration of each would be 1 uM/ml. Immediately after preparation, all solutions were distributed into 0.6 ml. quantities and stored in a deep freeze until used. Nucleotide triphosphates were obtained as Sodium salts from Cal Biochem and were dissolved in water.

Increase in TMV Infectivity Resulting From Incubation with Nucleotides.

Table 1. Increase in TMV Infectivity Resulting from Supplying Extracts from Infected Leaves with Triphosphate Nucleotides.

Expt. no.	Medium	Total Lesions			
		ATP,GTP, CTP,UTP	ATP,GTP CTP	ATP	None
4-27-62	Tris	90	49	___	___
4-28-62	Tris	91	53	___	___
4-30-62	Tris	280	112	___	___
5-2-62	Tris	460	___	___	374
5-7-62	Tris	1203	___	853	___
5-7-62	Phosphate	2630	___	2186	___
5-10-62	Phosphate	1324	___	1045	___
5-11-62	Phosphate	1492	___	1261	___

All incubations 1 hour at 21°. ___, not tested.

Table 1 presents the results of 8 separate experiments, each of which shows a rise in infectivity when infected leaf extracts are incubated with a combination of ATP, GTP, CTP, and UTP, and compared to incubations where 1, 3, or all 4 of the nucleotides are missing. The numbers are total lesions produced by the entire extracts applied to N. glutinosa leaves in the case of the first 4 experiments, or aliquots of the extracts in the last 4 experiments where extracts were produced by the method described. The first extracts were made by hand cutting of leaf tissue and produced small amounts of infectious material.

Immediately after incubation, the solutions were centrifuged, and the supernatants tested for infectivity. The pellets were resuspended in a volume of 0.05 M phosphate buffer equal to that of the supernatants and these suspensions were also tested for infectivity. The lesions from both pellets and supernatants have been combined in the table. In all cases the differences between the presence of the four

nucleotides and the absences of 1, 3 or all 4 are apparent in the separate testing of the pellets and supernatants. In the bioassay procedure, two experimental designs have been used. 1) Latin squares involving 4 levels per plant, with each sample being tested on not less than 8 leaves. 2) Half-leaf design where the top three, well-expanded leaves on several N. glutinosa plants were used. Two conditions are compared by alternating the treatments with respect to the right and left hand sides of a leaf with each comparison involving a minimum of 8, ½ leaf assays.

Sensitivity of Synthesized Infectivity to RNAase. Several experiments have indicated that addition of crystalline RNAase after the incubation period greatly reduces the amount of infectious material. The lesion counts in Table 2 illustrate this effect.

Table 2. RNAase Sensitivity of TMV-Infectivity Produced by an Extract.

Condition During Incubation	Total Lesions
ATP alone	1045
ATP followed by RNAase	66
ATP, GTP, CTP, UTP	1324
ATP, GTP, CTP, UTP followed by RNAase	103

RNAase treatment: 10 μg/ml, 1 hour incubation. The samples which did not receive RNAase were also subjected to an additional 1 hour incubation period. Latin square bioassay.

Time Course of TMV-RNA Synthesis by Extracts. The data presented in Table 3 show the effect of the time of incubation of extracts with the four nucleotide triphosphates on the amount of TMV infectivity. In experiment A, an extract was made from infected leaves as described, and divided into four equal aliquots at the 2nd wash. The pellets remaining after the third wash were suspended in incubation mixtures con-

taining the four nucleotides, and then incubated for various periods of time. The reaction was "stopped" by centrifuging the incubation mixture, and immediately inoculating N. glutinosa leaves with the supernatant solution.

In experiment B, an extract was made as in A but was not divided into aliquots. Instead, the pellet after the 3rd wash was resuspended in an incubation mixture containing the 4 triphosphate nucleotides. Aliquots were withdrawn from this mixture and immediately frozen after 0, 15, 30, and 60 minutes of incubation. The mixture was subjected to gentle shaking during incubation. After all of the samples had been collected and frozen, they were simultaneously thawed, centrifuged, and the supernatant solutions assayed for TMV infectivity.

Table 3. Time Course of Increase in TMV Infectivity by Extracts Supplied with Nucleotide Triphosphates.

Time of Incubation in Minutes	Number of Lesions Produced by Supernatant Solution	
	Expt. A	Expt. B
0	476	2024
15	609	2540
30	658	2830
60	820	2830

Incubation mixture No. 2 + 1.0 uM each of ATP, GTP, CTP, and UTP. Temperature 21°. Latin square bioassay.

Sensitivity of System to DNAase. The data in Table 4 show that the presence of DNAase in the incubation mixture prevents the rise in TMV infectivity produced by supplying the 4 triphosphate nucleotides.

Discussion. The question of whether DNA is an active or passive participant in this system is still not resolved. By analogy with other systems for synthesizing RNA, it is tempting to view the

Table 4. DNAase Sensitivity of TMV Infectivity System.

Condition	Total Lesions
1. Extract without nucleotides + DNAase	1269
2. Extract + 4 nucleotides + DNAase	1342
3. Extract + 4 nucleotides No DNAase	1896
4. Extract + 4 nucleotides DNAase added after incubation with nucleotides	2000

50 ug DNAase/ml; 3 x 10^{-3}M $MgCl_2$; Temp., 21°. 1 and 2, 1 hr. incubation with DNAase before 1 hr. incubation with or without nucleotides; 3 and 4, 1 hr. incubation with nucleotides before 1 hr. incubation with or without DNAase.

extracts from tobacco leaves as also containing hybrids of DNA and TMV-RNA which serve as templates for TMV-RNA synthesis. However, until further purification and characterization is accomplished, it could also be imagined that the DNA fibers merely serve to trap and hold other materials together long enough to permit synthesis of RNA. The levels of RNA synthesis would appear to range from about 0.5 to 2 ug equivalents of phenol prepared, TMV-RNA.

Acknowledgement. This work was supported by a grant, E-536(C8) from the National Institutes of Health, and Contract AT(11-1)-34, Project 8 from the Division of Biology and Medicine, Atomic Energy Commission. We have greatly benefited from the advice we have received from Professor Tokuzo Hirai of Nagoya University, Dr. J. H. Wu, and Mr. William Hudson.

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