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Turnip Yellow Mosaic Virus RNA as a Substrate of the Transfer RNA Nucleotidyltransferase

II. Incorporation of Cytidine 5'-Monophosphate and Determination of a Short Nucleotide Sequence at the 3' End of the RNA

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Turnip yellow mosaic virus (TYMV) RNA treated with snake venom phosphodiesterase accepts cytidine 5'-monophosphate and adenosine 5'-monophosphate (AMP) when it is incubated in the presence of cytidine 5'-triphosphate (CTP), adenosine 5'-triphosphate, and *Escherichia coli* transfer RNA nucleotidyltransferase; untreated TYMV RNA accepts only AMP. When α^{32}P-CTP was used for terminal labeling, the nearest neighbor analyses and the analyses after action of various nucleases showed that the sequence of five nucleotides at the 3' end of TYMV RNA is: pGpCpApCpC. A nuclease present in commercial preparations of snake venom phosphodiesterase leads to the fragmentation of TYMV RNA, the 3' end of which is found in a fragment having a sedimentation constant close to 5*s*.

The polynucleotide structure at the 3' end of turnip yellow mosaic virus (TYMV) RNA is recognized by at least four enzymes specific for transfer RNA (tRNA): valyl-tRNA synthetase, *N*-acyl aminoacyl-tRNA hydrolase (6, 7), tRNA nucleotidyltransferase (3), and the elongation factor EF-1 (Litvak et al., *in press*). When the RNA extracted from the virion by the phenol procedure is incubated in the presence of adenosine 5'-triphosphate (ATP) and *Escherichia coli* tRNA nucleotidyltransferase, one molecule of adenosine 5'-monophosphate (AMP) is accepted at the 3' end of the 23*s* RNA molecule. When α^{32}P-ATP was used for this reaction, the nearest neighbor analyses showed that the last nucleotide in TYMV RNA is cytidylic acid which is preceded by a pyrimidine nucleotide, probably also a cytidylic acid (3).

In order to gain more information on the tRNA-like structure of TYMV RNA, the 23*s* RNA molecule has been submitted to partial degradation by snake venom phosphodiesterase and checked for the acceptance of cytidine 5'-monophosphate (CMP) which is known to occur with -CCA-deprived tRNA. The incorporation of ^{32}P-CMP also offers the possibility of determining the sequence of several nucleotides at the 3' end of the TYMV RNA molecule.

During the course of this work we observed that, under the action of an endonuclease present in commercial preparations of snake venom phosphodiesterase, a TYMV RNA fragment is produced that has a sedimentation constant of about 5*s* and bears the 3' end of the viral RNA.

The biological significance of the presence of a tRNA-like structure at the 3' end of TYMV RNA is unknown. The fact that this structure appears to be recognized by various enzymes involved in protein synthesis suggests that it might play a specific role in the translation of the viral genome (Haenni et al., *in press*). It also might be a signal for the initiation of viral RNA replication.

MATERIALS AND METHODS

Materials. *E. coli* tRNA nucleotidyltransferase was purified as described previously (2). *E. coli* valyl-tRNA ligase was a gift of M. Yaniv, and total *E. coli* aminoacyl-tRNA synthetases were prepared

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as reported elsewhere (4). TYMV RNA was obtained from H. Duranton and from L. Bosch. To avoid degradation, the 23s RNA from both sources was kept as an ethanol suspension in the presence of bentonite. Snake venom phosphodiesterase and T_1 ribonuclease were purchased from Worthington Biochemical Corp., and U_2 ribonuclease was a gift of G. M. Barell. The radiochemicals α^{32}P-cytidine 5'-triphosphate (CTP), (400 mCi/mmole) and ^{14}C-valine (108 mCi/mmole) were from the Commissariat à l'Energie Atomique, Saclay (France). ^{3}H-CTP (1 mCi/μmole) and ^{3}H-ATP (1 mCi/μmole) were purchased from Schwarz-BioResearch. Purified $tRNA^{Phe}$ was a gift from B. F. C. Clark.

3' terminal labeling of TYMV RNA. The incorporation of CMP into TYMV RNA was performed as described previously for the incorporation of AMP (3). In a final volume of 0.2 ml the incubation mixture contained: 10 μmoles of tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (*p*H 8.5), 2 μmoles of $MgCl_2$, 0.2 μmoles of β-mercaptoethanol, 1 nmole of ^{3}H-CTP or α^{32}P-CTP, 50 pmoles of 3' terminal —pCpC-deprived TYMV RNA, and 5 to 10 μg of *E. coli* tRNA nucleotidyltransferase. After incubation, the RNA was precipitated with 5% cold trichloroacetic acid. After filtration on a Millipore filter and extensive washing with trichloroacetic acid, the radioactivity retained on the filter was determined by using a Nuclear-Chicago scintillation counter and Bray scintillation mixture.

Aminoacylation of TYMV RNA. When the incorporation of valine was tested, TYMV RNA was first preincubated in a mixture identical to that used for the tRNA nucleotidyltransferase assay, except that unlabeled ATP (0.1 μmole) was added. After 30 min at 37 C the *p*H was adjusted to 7 by addition of 10 μmoles of sodium cacodylate, *p*H 5.5.

To this mixture 3 nmoles of ^{14}C-valine and 1 μg of pure *E. coli* valyl-tRNA synthetase or 50 μg of a preparation containing all aminoacyl-tRNA synthetases were added, and the incubations were pursued for 30 min more. After precipitation with cold 5% trichloroacetic and filtration on a membrane filter (Millipore Corp.) the radioactivity retained was determined.

Snake venom phosphodiesterase treatment. The method used was similar to that described by Philipps et al. for tRNA (5). TYMV RNA (2 to 5 mg) was incubated in a final volume of 1 ml in the presence of 50 μmoles of glycine-NaOH (*p*H 8.5), 15 μmoles of $MgCl_2$, and 150 μg of snake venom phosphodiesterase (Worthington). After 15 min at 37 C the incubation mixture was filtered on a Sephadex G-25 column (45 by 1 cm), on top of which was a 1-cm thick layer of silicic acid; the column had been previously equilibrated with 0.1 M acetate buffer, *p*H 4.5. Before the incubation mixture was put onto the Sephadex column, 5 ml of a solution containing 10 mM Tris-hydrochloride (*p*H 7.3), 10 mM $MgCl_2$, and 0.3 M NaCl was adsorbed. The filtration was carried out by using 0.1 M acetate buffer, *p*H 4.5. Under these conditions the phosphodiesterase was retained on the silicic acid layer, the mononucleotides resulting from the exonuclease digestion were retarded on the Sephadex column, while the TYMV RNA was recovered in the void volume. TYMV RNA was concentrated by precipitation with two volumes of cold ethanol.

Alkaline hydrolysis of ^{32}P-CMP TYMV RNA. For each experiment 100 pmoles of TYMV RNA were used. ^{32}P-CMP was incorporated into TYMV RNA in the presence of tRNA nucleotidyltransferase as described above. The hydrolysis of the labeled RNA extracted by phenol and precipitated by ethanol was carried out for 14 hr at 37 C in 0.2 M KOH. The products of hydrolysis were analyzed by two-dimensional chromatography on silica gel Eastman type K 301 R sheets. For the first dimension the solvent used was propanol-NH_4OH-water (60:30:10) and for the second dimension, isopropanol-HCl-water (68:17.6:14.4).

Sephadex G-100 filtration of TYMV RNA. Five hundred picomoles of TYMV RNA treated by snake venom phosphodiesterase and filtered on a Sephadex G-25 column was labeled with ^{32}P-CMP in the presence of tRNA nucleotidyltransferase. The labeled RNA, precipitated by ethanol and dissolved in a 50 mM cacodylate buffer at *p*H 6 containing 0.1 M of NaCl, was filtered on a Sephadex G-100 column (1.2 by 116 cm). Optical density at 260 nm and radioactivity of the fractionated filtrate were determined.

Digestion of TYMV RNA in the presence of ribonucleases T_1 and U_2. The conditions were similar to those described by Barrel (*in press*). ^{32}P-CMP-labeled RNA (100 pmoles) was incubated in 0.15 ml with ribonuclease T_1 (weight ratio of enzyme/RNA = 1/20 in a solution containing 10 mM Tris-hydrochloride (*p*H 7.4) and 1 mM ethylenediamine tetracetic acid. After 30 min at 37 C the *p*H was brought to 4.5, and to the total volume of 0.2 ml, 10 μliters of a solution containing 0.1 unit of ribonuclease U_2 per ml and 1 mg of bovine serum albumin were added. The incubation was continued for 2 hr at 37 C. The fractionation of the hydrolysate was carried out by paper electrophoresis (at 4 C for 16 hr; 10 V/cm) on diethylaminoethyl (DEAE)-cellulose paper. The *p*H 1.9 buffer contained 2.5% formic acid, 8.7% acetic acid, and 88.8% water. After detection of radioactive spots by automatic scanning, the paper was cut into equal-sized pieces for a more precise radioactivity determination with Bray scintillation solution.

RESULTS AND DISCUSSION

CMP incorporation into phosphodiesterase-treated TYMV RNA. When TYMV RNA submitted to limited digestion by snake venom phosphodiesterase was incubated for 1 hr in the presence of ^{3}H-CTP and tRNA nucleotidyltransferase, it accepted approximately 1.5 moles of CMP per mole of RNA of molecular weight 2×10^6 (Fig. 1). TYMV RNA not treated with phosphodiesterase was a very poor acceptor of CMP. These results combined with those previously reported (3)

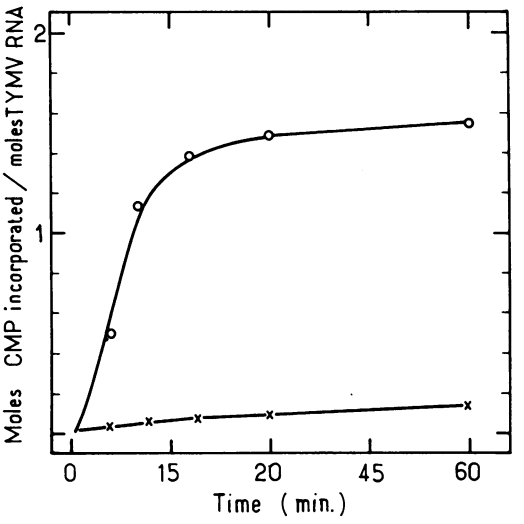

FIG. 1. *Kinetics of CMP incorporation into TYMV RNA. The RNA was incubated at 37 C in the presence of ^{3}H-CMP and E. coli tRNA nucleotidyltransferase as described in text. Symbols:* O, *TYMV RNA digested with snake venom phosphodiesterase;* ×, *TYMV RNA not digested.*

show that the tRNA nucleotidyltransferase-catalyzed addition of nucleotides to TYMV RNA is similar to that observed with tRNA since in both cases CMP and AMP are incorporated.

The study of the esterification of phosphodiesterase-treated TYMV RNA by valine in the presence of *E. coli* valyl-tRNA synthetase has shown that after addition of only CMP the RNA is unable to accept the amino acid (Table 1). After subsequent AMP addition, the extent of esterification of phosphodiesterase-treated RNA was about 15% lower than that observed with untreated RNA to which only AMP was added.

Nucleotide sequence at the 3′ end of TYMV RNA. The results reported previously have shown that the terminal cytidylate in TYMV RNA is preceded by a pyrimidine nucleotide (3). This nucleotide is also a cytidylate, as suggested by the fact that more than one molecule of CMP is incorporated into the phosphodiesterase-treated RNA. To obtain more information on this point, and at the same time on the nature of the third nucleotide, ^{32}P-CMP-labeled RNA was submitted to alkaline hydrolysis, and the 2′-3′ nucleotides formed were analyzed by two-dimensional thin-layer chromatography. It was found that only CMP and AMP contained ^{32}P. This result clearly shows that the second nucleotide from the 3′ end is a cytidylate as expected, and the third is an adenylate.

The nature of the fifth nucleotide was deduced from results published by Yot et al. (7). When ^{14}C-valyl-RNA of TYMV was submitted to ribonuclease T_1 digestion and the product was chromatographed on DEAE-cellulose in the presence of 7 M urea, it was found that valine was attached to the 3′ adenosine of a pentanucleotide; this means that the fifth nucleotide preceding the 3′ terminal cytosine (or the sixth, preceding 3′ adenosine after addition of AMP) is a guanylate. The sequence can then be written: ...pGpX-pApCpC(pA).

The nature of the fourth nucleotide was determined by hydrolysis of ^{32}P-CMP-labeled TYMV RNA with ribonucleases T_1 and U_2 and analysis of the labeled products. Since T_1 is specific for guanine and U_2 for adenine, the product should be either Ap or XpAp. The use of pancreatic ribonuclease showed that X is a pyrimidine. To determine the nature of X, yeast $tRNA^{Phe}$, which has the 3′ terminal structure pGpCpApCpCpA, was chosen to prepare the CpAp dinucleotide. Both XpAp and CpAp labeled in their 3′ terminal phosphate were obtained by the same method. The comparison of their behavior on paper electrophoresis showed (Fig. 2) that they are identical and consequently that the sequence of five 3′ terminal nucleotides of TYMV RNA is pGpCpApCpC.

TABLE 1. *Esterification of TYMV RNA with valine*[a]

TYMV RNA	Incorporation (moles/mole of RNA)		
	CMP	AMP	Valine
Untreated			0.03
Untreated	0.08	0.63	0.69
Phosphodiesterase treated			0.02
Phosphodiesterase treated	1.38		0.02
Phosphodiesterase treated	1.38	0.61	0.58

[a] Incorporations were carried out in the presence of highly purified enzymes. The valyl-tRNA synthetase was free of nucleotidyltransferase activity. For the determination of AMP incorporation the RNA was first incubated in the presence of CTP for 20 min and then after addition of ^{3}H-ATP for an additional 20 min. For each esterification with valine, 50 pmoles of RNA was used. Other conditions were those described in Materials and Methods.

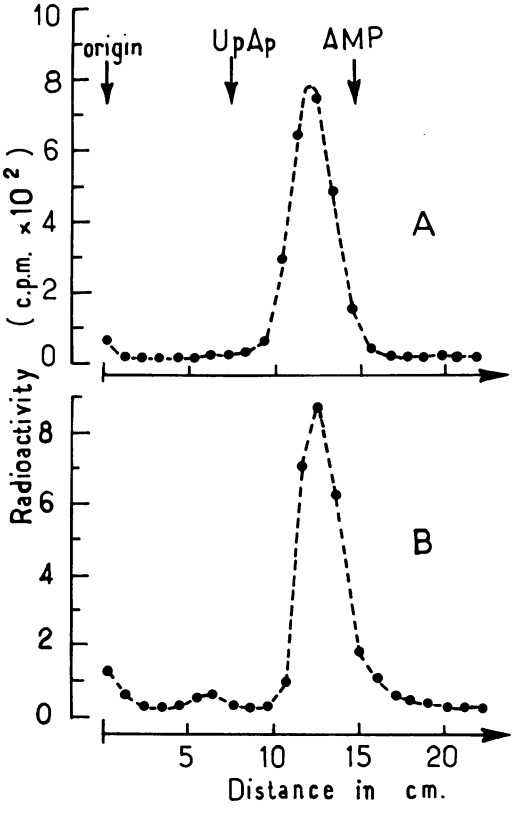

FIG. 2. *Characterization of the fourth nucleotide from the 3' end of TYMV RNA. Paper electrophoresis of the T_1 and U_2 ribonuclease products of either TYMV RNA (A) or of tRNA^{Phe} (B) labeled with ^{32}P-CMP in the presence of tRNA nucleotidyltransferase. Incubations and conditions of analysis are as described in text.*

The 3' fragment of TYMV RNA as substrate for the tRNA nucleotidyltransferase. During the sequence work which is reported in this paper it was found that incubation of TYMV RNA in the presence of commercial preparations of snake venom phosphodiesterase leads not only to the removal of the 3' terminal cytidylates but also to a fragmentation of the 23*s* RNA molecule. This is probably due to the presence in these preparations of a contaminating endonuclease. The interesting feature of this endonucleolytic splitting is that a fragment of about 5*s* from the 3' end representing approximately 2% of the total RNA molecule is liberated almost quantitatively without being degraded further. It was checked that under the same conditions the tRNA is not fragmented. Three different methods used for the characterization of the size of this fragment gave similar results: glycerol gradient centrifugation, polyacrylamide gel electrophoresis, and Sephadex G-100 filtration. This fragment is used as a substrate by the tRNA nucleotidyltransferase, and Figure 3 shows the filtration profile of the ^{32}P after incubation of phosphodiesterase-treated TYMV RNA in the presence of the enzyme and α^{32}P-CTP.

Yot et al. (7) have observed that, after TYMV RNA is heated and incubated in the presence of *E. coli* extracts, an important part of RNA molecules furnished a 3' fragment of 4 to 5*s*. More recently Prochiantz and Haenni (*in press*) found that the *E. coli* endonuclease specific for the removal of a polynucleotide fragment from the precursor tRNA molecules during maturation (1) also acts on TYMV RNA and leads to the formation of a 4.5*s* fragment from the 3' end.

Two interpretations can be given as to the mode of action of the contaminating endonuclease present in the phosphodiesterase preparations. Either the enzyme is similar to that of the tRNA maturation nuclease, or most probably it is a nonspecific nuclease; but, at the concentrations at which it was used, a tRNA-like structure at the 3' end of the TYMV RNA molecule is resistant to the enzyme. Further experiments are needed to clarify this point.

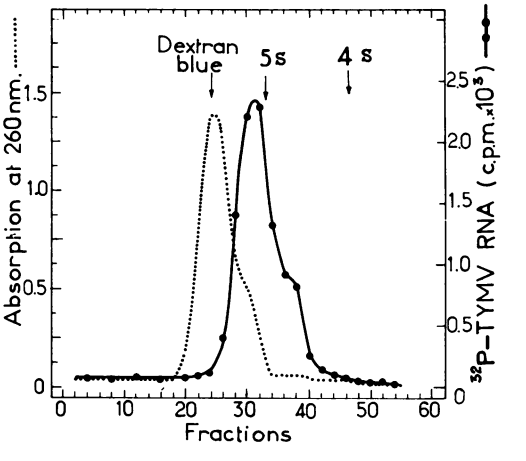

FIG. 3. *Gel filtration of phosphodiesterase-treated TYMV RNA. After action of snake phosphodiesterase (Worthington), the RNA was isolated and incubated in the presence of α^{32}P-CTP and tRNA nucleotidyltransferase, and the product was filtered on a Sephadex G-100 column. The conditions are described in text. It was checked that after addition of CMP the 5s fragment was also an acceptor of AMP and of valine.*

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