RECOGNITION OF TMV RNA BY THE tRNA NUCLEOTIDYLTRANSFERASE

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1. Introduction

Within the last few years, evidence has accumulated that several plant viral RNA's possess a tRNA-like structure at their 3′ end, since they can be charged with an amino acid, and since they are recognized by a series of enzymes specific of tRNAs [1–9]. Thus, the RNA of Turnip Yellow Mosaic Virus (TYMV) and of two other tymoviruses can be aminoacylated with valine [1,2,7], and the RNA of the members of the bromovirus group and that of Cucumber Mosaic Virus by tyrosine (8 and R. J. Kohl and T. C. Hall, personal communication).

It has been reported that TMV RNA can accept histidine [4,9], and moreover that the histidyl-RNA can form a ternary complex with wheat embryo elongation factor EF-1 and GTP [4]. Further, the sequence of the pentanucleotide at the 3′ end of the viral RNA of 2×10^6 daltons [10] is known to be –GCCCA [11]. It is interesting to note that only few tRNAs contain a cytidine residue in the fourth position from the 3′ end [12]. Those which are known are acceptor only of histidine or of proline.

Because of the tRNA-like properties of this viral RNA, and since it contains the 3′ terminal sequence –CCA common to all tRNAs, we have investigated whether it could also be a substrate of the tRNA nucleotidyltransferase (CCA-enzyme), the enzyme capable of adding back –CCA to tRNA deprived of this 3′ terminal sequence.

Our conclusion is that under conditions wherein the CCA-enzyme 'repairs' tRNAs, it also adequately 'repairs' TMV RNA, adding back the –CCA sequence to the RNA from which the 3′ terminal trinucleotide has been previously removed. As this work was nearing completion, we learned that similar results had been obtained by S. Litvak (personal communication).

2. Materials

The following strains of TMV: Vulgare, U_2, HRG and Dahlmensis were kindly provided by Dr. S. J. Morris (Heidelberg), and their viral RNA's extracted as described by Gierer and Schramm [13]. Highly purified *E. coli* tRNA nucleotidyltransferase was a kind gift of Dr. D. Carré. We are grateful to Dr. C. Gualerzi for a sample of rat liver high speed supernatant devoid of $tRNA^{His}$. Snake venom phosphodiesterase was from Worthington and Ribonuclease T_1 from Sankyo. [^{3}H]ATP (22.7 Ci/mmole) and [^{14}C]-CTP (308 mCi/mmole) were from Schwartz Bio-Research, and [^{14}C]histidine (45 mCi/mmole) from the Commissariat à l'Energie Atomique (Saclay).

3. Methods

3.1. *Esterification of histidine to TMV RNA*

The incubation mixture contained in a final volume of 0.2 ml: 10 μmoles Tris–HCl pH 7.4, 2 μmoles $MgCl_2$, 2 μmoles dithiothreitol, 0.4 μmole ATP, 150 μg of rat liver high speed supernatant, 150 μg of TMV RNA and 0.01 μmole of [^{14}C]histidine. After incubation for 20 min at 37°C, 10% cold trichloroacetic acid was added, the precipitate collected on Millipore filters and counted in Bray's solution.

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3.2. *Treatment of TMV RNA with snake venom phosphodiesterase*

The conditions of incubation were analogous to those described by Miller et al. for tRNA [14]. In a final volume of 0.1 ml containing 10 μmoles Tris–HCl pH 7.7, 1 μmole Mg acetate and 30 μg of snake venom phosphodiesterase, 200 μg of TMV RNA were added and incubation performed for 15 min at 37°C. The reaction was stopped by addition of an equal volume of water-saturated phenol, and the RNA's recovered from the aqueous phase by ethanol precipitation.

3.3. *Incubation with the CCA-enzyme*

The experimental conditions were similar to those of Carré et al. [15]. The incubation was performed in a final volume of 0.05 ml containing 2.5 μmoles Tris-acetate pH 8.3, 0.5 μmole Mg acetate, 0.5 μmole DTT, 0.25 nmole CTP, 2.5 nmoles [^{3}H]ATP (1.25 Ci/mmole), 75 μg of TMV RNA previously treated with or without snake venom phosphodiesterase, and 1–2 μg of CCA-enzyme. Incubation was for 1 to 3 hr at 37°C. The reaction was stopped by addition of cold 10% trichloroacetic acid. The precipitates were collected on Millipore filters and their radioactivity determined in the presence of Bray's solution.

In experiments where labelled CTP was also used, 0.8 nmoles [^{14}C]CTP replaced the unlabelled CTP and a preincubation step was performed in the presence of all the other ingredients except ATP for 20 min at 37°C. [^{3}H]ATP was then added at the concentrations indicated and incubation pursued 20 min more. The reaction was stopped by addition of cold 10% trichloroacetic acid, and the radioactivity in the precipitates determined as above.

For preparative purposes the incubation mixtures were scaled up and the incubation performed as above, except that unlabelled CTP (2.5 nmoles/0.5 ml incubation mixture) was used and that the specific activity of the [^{3}H]ATP was 750 mCi/mmole. The reaction was stopped by addition of phenol and the labelled product was recovered by ethanol precipitation. It was then purified by filtration over a Sephadex G-100 (0.9 × 30 cm) column equilibrated with 0.01 M sodium cacodylate pH 6.0, 0.1 M NaCl and 0.02 M EDTA. The material eluting in the void volume was collected and concentrated by ethanol precipitation.

3.4. *DEAE-cellulose chromatography of the RNase T_1 digests of [^{3}H]AMP RNA of TMV*

The incubation conditions and the procedure for the analysis of the labelled oligonucleotide by DEAE-cellulose chromatography were as already described [2]. TMV RNA (200 μg containing 30 pmoles of [^{3}H]AMP) were incubated with 50 units of RNase T_1 in a total volume of 300 μl containing 15 μmoles Tris–HCl pH 7.7 and 6 μmoles EDTA. After 15 min at 37°C, the mixture was adjusted to 0.05 M ammonium acetate pH 5.5 and 7 M urea, and loaded on a DEAE-cellulose column (15 × 0.9 cm) equilibrated in the same solution. After washing of the column, a linear (0.05–0.6 M) ammonium acetate gradient pH 5.5 in 7 M urea was applied (total volume = 150 ml). One ml fractions were collected and counted in the presence of 10 ml Triton-fluor.

4. Results and discussion

4.1. *AMP incorporation into various strains of TMV RNA*

It has been reported that the level of aminoacylation using a yeast extract as source of histidyl-tRNA synthetase varies from one strain of TMV RNA to another; further, it has been proposed that the tRNA-like structure of the TMV RNA's which are poorly aminoacylated might be embedded in the viral genome and thus not be readily accessible to the histidyl-tRNA synthetase, since preheating of

Table 1
Comparison of histidine and AMP incorporation into various strains of TMV RNA

Strain	pmoles histidine bound per pmole untreated RNA	pmoles AMP bound per pmole phosphodiesterase-treated RNA
U_2	0.25	0.30
Vulgare	0.24	0.40
HRG	0.24	0.18
Dahlmensis	0.04	0.19

The conditions were given under Methods. A level of 0.05 pmole AMP was bound per pmole of untreated TMV RNA.

such RNAs leads to an increase in histidine acceptance [16].

We have therefore investigated whether a similar difference between strains could be detected with the CCA-enzyme. Having confirmed that incorporation of AMP with the CCA-enzyme was negligeable when untreated TMV RNA was used as substrate [17], the viral genome was treated with snake venom phosphodiesterase prior to incubation with the CCA-enzyme. The results presented in table 1 show that there was no direct correlation between the level of AMP incorporation and aminoacylation; they confirm the fact that with a rat liver extract also, however, the amount of histidine bound to the RNA depends on the TMV strain used.

4.2. *Effect of CMP incorporation on the incorporation of AMP into TMV RNA*

In order to verify whether the incorporation of AMP was dependent on the incorporation of CMP, phosphodiesterase-treated TMV RNA was preincubated in the presence or absence of CTP, after which [^{3}H]ATP was added and incubation pursued. The data presented in fig. 1 show that indeed incorporation of AMP was favored by preincubation with CTP. The AMP incorporated in the absence of CTP may reflect insufficient treatment of the RNA with snake venom phosphodiesterase that would have led to RNA molecules deprived only of the terminal adenosine.

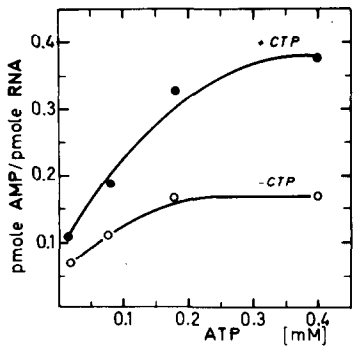

Fig. 1. Effect of CMP incorporation on AMP incorporation into TMV RNA (Vulgare). Conditions were as described under Methods, control experiments being performed in the absence of CTP. The specific activity of the [^{3}H]ATP was decreased for high concentrations of the nucleotide: it varied between 2 and 0.4 Ci/mmole for concentrations between 0.01 and 0.4 mM respectively. It was previously verified by kinetic studies that in the conditions of CTP concentration used here, incorporation of CMP reached a plateau level after 20 min of incubation at 37°C. This level correspond to 0.8 pmole of CMP/pmole of RNA, or 40% incorporation if it is assumed that two CMP residues are incorporated per RNA molecule.

Fig. 2 presents the results of an experiment in

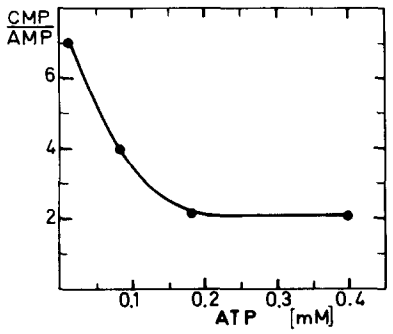

Fig. 2. Ratio of CMP to AMP incorporation into TMV RNA (Vulgare) as a function of ATP concentration. Experimental conditions were as indicated under Methods, and the specific activities of the [^{3}H]ATP as given in the legend of fig. 1.

which incorporation of [^{14}C]CMP and [^{3}H]AMP were measured with increasing [^{3}H]ATP concentrations. At a concentration of $1 \times 6 \times 10^{-5}$ M CTP, 2×10^{-4} M ATP is required to reach the stable value of two CMP incorporated per AMP, as also reported by Carré et al. [15] for tRNA's. Our results therefore suggest that incorporation into phosphodiesterase-treated TMV RNA proceeds by the successive addition of two CMP and one AMP residues into the 3′ terminus of the viral genome.

4.3. *RNase T_1 digests of TMV RNA bearing [^{3}H]AMP*

If indeed the CCA-enzyme incorporated CMP and AMP into the 3′ end of the viral RNA, then after RNase T_1 digestion, analysis by DEAE-cellulose chromatography should reveal a tetranucleotide corresponding to the 3′ terminus of the RNA, since TMV RNA is terminated by the sequence –GCCCA [11].

Phosphodiesterase-treated TMV RNA was incubated with the CCA-enzyme as indicated in Methods, using unlabelled CTP and [^{3}H]ATP. After RNase T_1 digestion, the oligonucleotides were separated by DEAE-cellulose chromatography. The elution profile of the labelled oligonucleotides is presented in fig. 3.

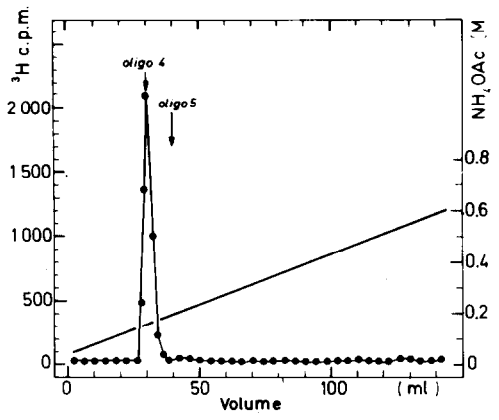

Fig. 3. Analysis of the Rnase T_1 digests of TMV RNA (U_2) bearing [^{3}H]AMP. The incubation condition and the analytical procedure were as described under Methods.

Only a single peak is detected, whose position on the column is in accordance with that of a tetranucleotide, since it coincides with that of TMV histidyl-RNA treated under similar conditions [16], and since it elutes before TYMV [^{3}H]valyl-RNA or TYMV [^{3}H]AMP-RNA obtained by a similar treatment which yields a pentanucleotide ([2] and A. Prochiantz et al., in preparation).

These results therefore indicate that the CCA-enzyme has added back CMP and AMP into the 3′ terminus of the phosphodiesterase-treated TMV RNA.

Because tRNA nucleotidyltransferase has been found in all cells examined to date, it is undoubtedly present in the host cells of TMV. Consequently, since TMV RNA is a substrate of the tRNA nucleotidyl transferase, one can imagine that this enzyme may play a role in the synthesis of the viral RNA by terminal addition of AMP and possibly CMP after replication.

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