Methylation of TMV RNA*

K. Marcu and B. Dudock

Department of Biochemistry
State University of New York at Stony Brook
Stony Brook, New York 11794

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Several plant and animal viral RNAs contain a tRNA like structure at their 3' ends. In this communication we show that tobacco mosaic virus (TMV) RNA is an acceptable substrate for a specific tRNA methyltransferase. Using a crude preparation of E. coli ribothymidine (rT) forming uracil methylase and (methyl ^{3}H) S-adenosyl-L-methionine (SAM) as a methyl donor, 0.7 moles of methyl group is incorporated per mole of TMV RNA in 10 hours at 30°C. Upon T_2 RNAse digestion of the labeled RNA, all of the radioactivity was found to be in TMP. T_1 RNAse digestion of ^{3}H methylated TMV RNA showed that all of the label was located in a tetranucleotide which co-migrated with authentic TpψpCpGp, an oligonucleotide characteristically found in normal cellular tRNA.

The use of this specific methyl transferase reaction may provide a simple assay for the detection of tRNA like structures in large RNAs.

Much information is now available on the existence of tRNA like structures located at the 3' end of several plant and animal viral mRNAs. The viral RNAs from TMV (1-3), BMV (4), TYMV (5,6), eggplant mosaic virus (7), okra mosaic virus (7) and mengovirus (8) are acceptable substrates of aminoacyl tRNA synthetases. E. coli tRNA nucleotidyl transferase (9), N-acylaminoacyl-tRNA hydrolase (6), and a partially purified preparation of E. coli RNAse "P" (a tRNA processing endoribonuclease) (10) have shown to effectively react

Abbreviations:

TMV (Tobacco mosaic virus), BMV (Brome mosaic virus), TYMV (Turnip yellow mosaic virus), DEAE cellulose (Diethylaminoethyl cellulose), SAM (S-adenosyl-L-methionine), rT (ribothymidine), EF (Elongation Factor), m^5s^2U (5-methyl-2-thiouracil), TMP (ribothymidylic acid).

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with TYMV RNA. Similarly, the 3' end of phosphodiesterase treated TMV RNA has been repaired by the addition of a CpCpA$_{OH}$ sequence by tRNA nucleotidyl transferases (11). RNAse "P" is capable of yielding a 4.5s RNA fragment from TYMV RNA which is an acceptable substrate of E. coli valine tRNA synthetase (10). In addition, elongation factor 1 from wheat embryos forms a ternary complex with TYMV val-RNA or TMV his-RNA in the presence of GTP (2).

Studies on the possible function of these tRNA like structures in protein biosynthesis are inconclusive at present. The transfer of valine esterified to the 3' end of TYMV RNA into protein has been achieved with a synthetic messenger directed E. coli protein synthesizing system (12). However, no substantial incorporation of tyrosine by BMV tyr-RNA has been obtained in a BMV directed wheat embryo protein synthesizing system (13). The oxidation of the 3' end of BMV RNA has no apparent effect on in vitro protein synthesis directed by BMV RNA in the wheat embryo system (14).

In this communication, we report on the methylation of TMV RNA with an E. coli rT forming uracil methylase and (methyl ^{3}H) SAM as the methyl donor. Using the described reaction conditions, 0.7 moles of methyl group is incorporated per mole of TMV RNA to form ribothymidine (assuming the molecular weight of TMV RNA is 2×10^{6} daltons) (15). An analysis of the labeled material following T_1 RNAse digestion indicates all the label is incorporated into a tetranucleotide with the chromatographic mobility of T-Ψ-C-Gp. The nucleotide sequence T-Ψ-Cp has been found to be common in almost all tRNAs (with few exceptions) (16-24) which function in the elongation step of protein biosynthesis and has been implicated as a ribosome binding site interacting with ribosomal 5s RNA (36).

Materials and Methods

The preparation of rT forming uracil methylase from E. coli MRE 600 has been described (22,24). The methylation

reaction was performed at 30°C in 50 μl at a TMV RNA concentration of 100 μg/ml; with 13.4 μM (methyl ^{3}H) S-adenosyl-L-methionine; 50 mM phosphate pH 8.2, 1 mM EDTA; 1 mM $MgCl_2$ and approximately 70 μg of crude methylase.

TMV RNA (variety vulgare) was prepared by the procedure of Marcus, Efron and Weeks and stored in liquid N_2 to maintain stability (25).

Methylation assays were terminated by the addition of 1 ml of 10% TCA unless otherwise noted. The precipitates were collected onto Whatman GF/A filters and counted in Omniflour-Toluene in a Nuclear Chicago Liquid Scintillation counter with an ^{3}H efficiency of 45%. The ^{14}C and ^{3}H counting efficiencies for double label experiments were 80% and 20%, respectively. Radioactive column effluents were added to Aquasol (200λ in 2 ml and 400λ in 4 ml of Aquasol) obtained from New England Nuclear and counted in either a Beckman or a Nuclear Chicago Liquid Scintillation counter both with ^{3}H and ^{14}C efficiencies of 45% and 95%, respectively.

Thin layer chromatography was performed on Eastman chromogram cellulose sheets and developed as described (24).

(methyl ^{3}H) SAM with a specific activity of 8.5 c/mmole and (methyl ^{14}C) SAM with a specific activity of 55.5 μc/μmole were purchased from New England Nuclear and ICN, respectively.

RNAses T_1 and T_2 were purchased from Calbiochem and the separation of digestion products on DEAE cellulose has been described in detail elsewhere (26).

Results and Discussion

TMV RNA was found to be an acceptable substrate of a crude preparation of E. coli rT forming uracil methylase with (methyl ^{3}H) SAM as the methyl donor. In 10 hours at 30° C, 0.7 moles of methyl group is incorporated in a reaction containing 2.5 pmoles of TMV RNA (assuming a molecular weight of 2×10^6 daltons for TMV RNA) (15). The reaction reaches a plateau in 10 hours as seen from the reaction kinetics shown in Fig. 1. Following T_2 RNAse digestion of the methylated TMV RNA all of the radioactivity incorporated was found to be TMP by two dimensional thin layer chromatography (22).

Ribothymidine is a very widely found modified nucleoside located at the 23rd position from the 3' end of almost all elongator tRNAs sequenced to date. It is not however found in Bacillus subtilis tRNAs (17), the tRNAs of an E. coli mutant defective in rT synthesis (21) , Mycobacterium lysodeictus tRNAs (18), Mycoplasma

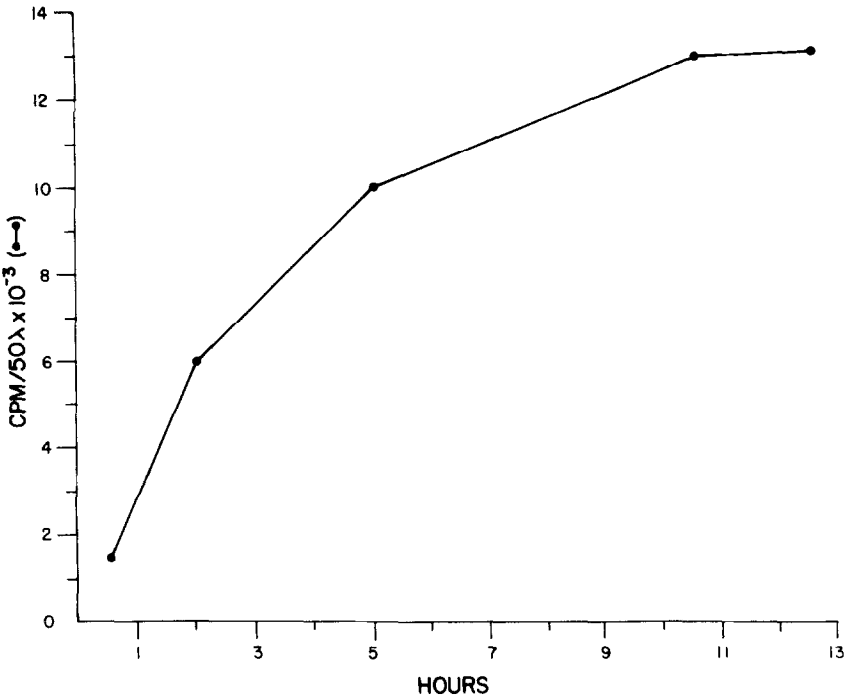

Figure I

A methylation reaction containing 5 μg of TMV RNA and (methyl ^{3}H) SAM as described in materials and methods was incubated at 30°C and 5μl aliquots were taken at the times indicated and precipitated with approximately 0.5 ml of 10% TCA. The precipitates were collected on GF/A filters washed and counted. The radioactivity incorporated in a corresponding control reaction without TMV RNA for each of the time points selected was subtracted from the methylation reaction with TMV RNA. The resulting counts were corrected for the total amount of radioactivity incorporated in 50 μl.

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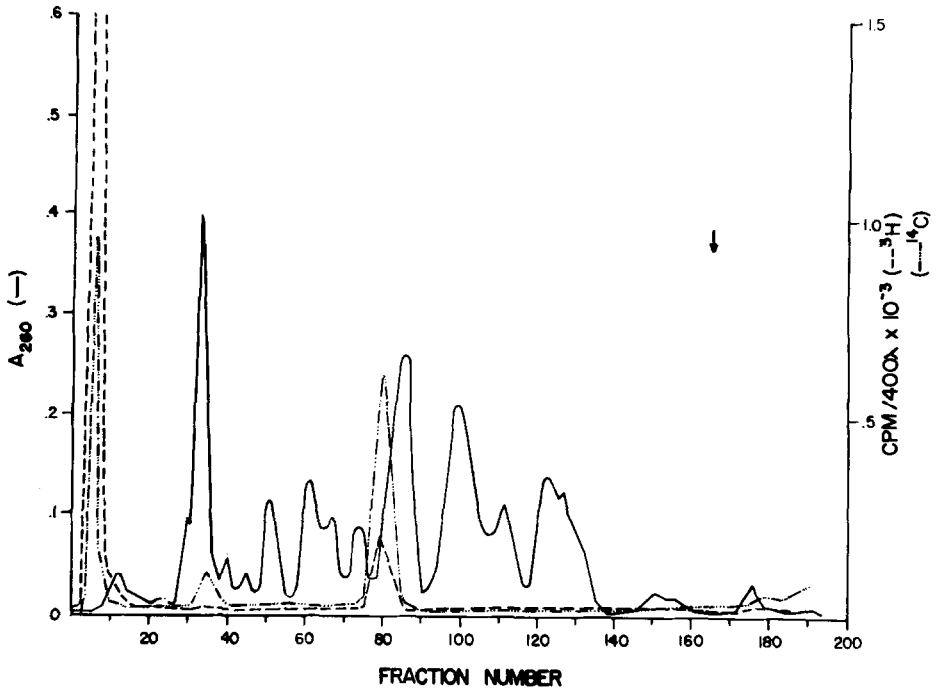
tRNAs (16), wheat germ glycine and threonine tRNAs (22), rabbit liver $tRNA_3^{Glu}$ and $tRNA_2^{Lys}$ (23) and specific bovine and fetal calf tRNAs (31). Adenosine replaces rT in the eukaryotic initiator tRNAs (27-30) and a modified rT is found in Flavobacterium thermophilum where m^5s^2U replaces m^5U (rT) (19) and rabit liver $tRNA_3^{Lys}$ which has a 2'-O methyl m^5U (20). In addition, recent studies on hamster-cell mitochondrial tRNAs have shown a low rT content (32), and ^{3}H-postlabeling experiments on 4S RNA of several rat tissues have indicated that approximately 50% of unfractionated cytoplasmic tRNAs lack rT (33). Mycoplasma (16), Bacillus subtilis (17) and



wheat germ glycine and threonine tRNAs (22) have been shown to be acceptable substrates of E. coli rT forming uracil methylase. We have previously shown that T_1 RNAse digestion of methylated wheat germ $tRNA_1^{Gly}$ releases T-ψ-C-Gp as a labeled tetranucleotide (22). A similiar analysis was performed on methylated TMV RNA in the present study.

A mixture of ^3H methylated TMV RNA and ^{14}C methylated $tRNA_1^{Gly}$ was digested with T_1 RNAse and the resulting products were chromatographed on a DEAE cellulose column (Fig. 2). The ^{14}C peak at tube 79, which is T-Ψ-C-Gp was found to co-migrate exactly with the ^3H peak, both of which elute well ahead of the peak containing U-Ψ-C-Gp (tube 84) (resulting from excess $tRNA_1^{Gly}$ added as marker and carrier). This data strongly suggests that a tetranucleotide similiar or identical to U-Ψ-C-Gp or U-U-C-Gp exists in TMV RNA and that this sequence is methylatable with E. coli rT forming uracil methylase.

Much evidence has accumulated on the involvement of T-Ψ-C-Gp in the binding of tRNAs to ribosomes. Studies conducted by Ofengand and Henes have shown that T-Ψ-C-Gp inhibits the nonenzymatic binding of aminoacyl tRNA to 70S ribosomes (34). Recently, Schwarz et al. (35) have shown that in the presence of C-G-A-A (a sequence complimentary to T-Ψ-C-Gp) in vitro polyphenylalanine synthesis was inhibited 40%, while the tetranucleotide A-A-A-A apparently had no effect. Erdmann et al. have shown that T-Ψ-C-Gp specifically binds to 5S RNA and this binding is enhanced 10 fold in the presence of specific ribosomal proteins (36). Further studies have implicated

Figure 2

A methylation reaction containing 5 µg of TMV RNA and the appropriate concentration of (methyl ^{3}H) SAM as described in materials and methods was incubated at 30°C for 10 hours. This reaction was pooled with another methylation reaction containing 0.5 mgs of wheat germ $tRNA_I^{Gly}$ and (methyl ^{14}C) SAM which was incubated at 30°C for 5 hours in a total volume of 1 ml (22) and 10 drops of H_2O saturated phenol was added to terminate the reactions. The mixture was shaken vigorously and then centrifuged at top speed in an IEC table top centrifuge for approximately 3 min. The aqueous phase was removed with a pasteur pipette and the interphase and phenol phase were reextracted with 200 µl of 0.05M Tris (pH 7.5). The aqueous phases were combined and the RNA precipitated with two volumes of 95% ethanol and several drops of 2M potassium acetate. After allowing it to stand at -20°C for 1 hour, the material was centrifuged and the pellet washed several times with 95% ethanol. After lyophilization, the dried material was resuspended in 300 µl of 0.05 M Tris (pH 7.5) and digested with 250 units of T_1 RNAse for four hours at 37°C. The reaction was applied to a DEAE Cellulose (carbonate) column (0.4 cm x 54 cm) equilibrated in 0.05 M $(NH_4)_2CO_3$ (pH 8.8). A linear gradient 0.05M to 0.50M $(NH_4)_2CO_3$ (pH 8.8) (100 ml each chamber) was generated. The column was run at 50 lb/inch^2 with a Milton Roy Pump at a flow rate of 1.15 ml/min. The arrow indicates the addition of a 1M $(NH_4)_2CO_3$ wash. The labeled material eluting between fractions 2 and 10 is SAM. The ^{14}C radioactivity (due to $tRNA_I^{Gly}$) eluting between fractions 30 and 40 was previously found to be $T-G_p$ (22). Similarly, the labeled fragment eluting between fractions 75 and 85 was previously shown to be $T-\psi-C-G_p$ (22). The optical density pattern is a characteristic T_1 fingerprint of wheat germ $tRNA_I^{Gly}$ obtained under these conditions (39). Radioactive column effluents were counted as described in materials and methods.

In a separate experiment ^{3}H methylated TMV RNA was digested with T_1 RNAse and chromatographed on DEAE cellulose exactly as described above, but in the absence of ^{14}C methylated $tRNA_I^{Gly}$ as a marker. The ^{3}H labeled tetranucleotide was found to eluate in the same position as in the double-label experiment, thus eliminating the possibility that the tritium peak at tube 79 was due to "spillover" from the ^{14}C channel during liquid scintillation counting.

two specific adenines in 5S RNA in the binding of T-Ψ-C-G (36). In addition, recent studies on T-Ψ-C-Gp have indicated that this nucleotide sequence in tRNAs is involved in ppGpp and pppGpp formation in E. coli (37,38).

Although the tRNA like structure at the 3' end of TMV RNA is certainly the most likely position for the methylation reaction, the precise location of methylation has not been determined at the present time. After 5 hours of reaction the incorporated ^{3}H methyl appears to be in a range of fragments due to ribonucleases present in the crude methylase preparation (Fig. 3a). As expected, unreacted TMV RNA elutes in the void volume of a Sephadex G-100 column and 25% of the methylated RNA continues to elute in the void volume, while approximately 30% of the label migrates as a fragment slightly smaller than the size of tRNA. G-100 chromatography after 10 hours of reaction now shows no labeled material eluting in the void volume while 70% of the label elutes as a nucleotide fragment slightly smaller than a tRNA molecule of 74 nucleotides in length (Fig. 3b). The determination of the site of methylation must await purer preparations of E. coli rT forming uracil methylase which are essentially free of ribonucleases.

In addition to the methylation of TMV RNA with a tRNA methyltransferase as described in this report, TMV RNA has been previously shown to be an acceptable substrate of the histidine aminoacyl-tRNA synthetases from yeast, wheat and E. coli (2) and is capable of forming a ternary complex in the presence of wheat EF-1 and GTP (2) and is also an acceptable substrate of a tRNA nucleotidyl transferase (11). Studies on TMV strains U_2 and Vulgare have indicated an acceptor activity of .26-.30 moles of histidine per mole of RNA, while strain dahlmensis was apparently totally unchargeable (2). Studies on the aminoacylation of TMV Vulgare with histidine using a preparation of wheat germ

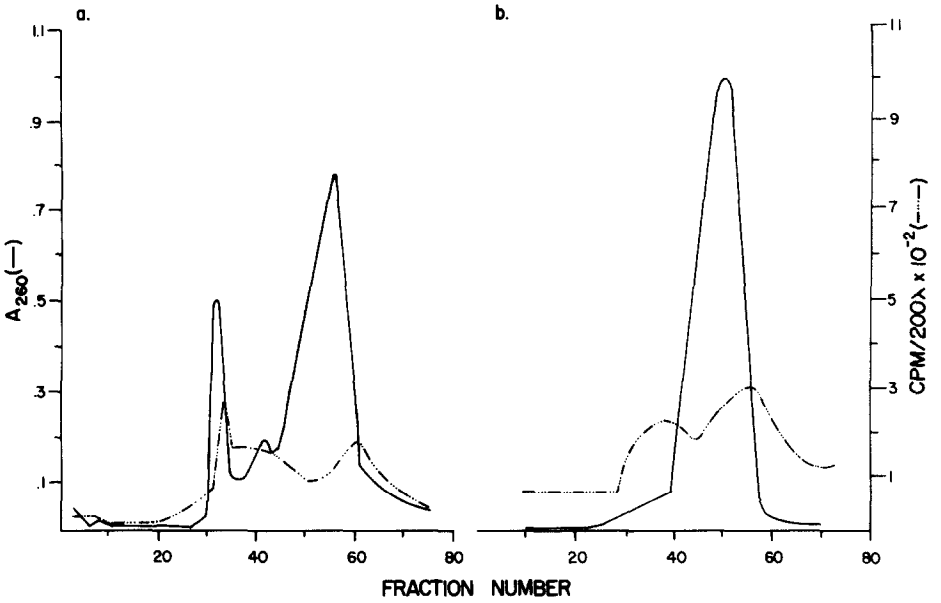


Figure 3

(a.) Two methylation reactions each containing 5 µg of TMV RNA were incubated for 5 hrs at 30°C as described in materials and methods. The reactions were pooled and terminated by the addition of 8 drops of H_2O saturated phenol and 250 µg of crude wheat germ tRNA in 400 µl was added as carrier. The mixture was shaken vigorously and then centrifuged at top speed in an IEC table top centrifuge for approximately 3 min. The aqueous phase was removed with a pasteur pipette and the interphase and phenol phases were reextracted with 200 µl of 0.05M Tris (pH 7.5). The aqueous phases were combined and the RNA precipitated with two volumes of 95% ethanol and several drops of 2M potassium acetate. After lyophilization, the dry material was resuspended in 100 µl of Sephadex G-100 buffer (0.1M NaCl, 0.01M $MgCl_2$, 0.010M Tris pH 7.5) and applied to a Sephadex G-100 column (1 cm x 115 cm) along with 40 µg of unmethylated TMV RNA. 400 µl was collected per fraction in 3.6 min. The first optical density peak is TMV RNA eluting in the void volume and the second, 5S RNA and the third 4S RNA.

(b.) The product of two methylation reactions were again applied to a Sephadex G-100 column as described in (a.) but in this case: the reactions were incubated for 10 hours and 250 µg of pure wheat germ $tRNA^{Gly}_1$ was added as carrier and marker (74 nucleotides in length) (39) instead of crude wheat germ tRNA. 475 µl was collected per fraction in 2 min.

Radioactive column effluents in (a.) and (b.) were counted as described in materials and methods.

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synthetase in our laboratory has resulted in a comparable level of aminoacylation (.3 mole his/mole TMV RNA). The levels of amino-acylation of all viral RNAs which possess a tRNA like 3' end tested

to date have been considerably less than 1 mole of a specific amino acid incorporated per mole of RNA (1-8). If these specific viral RNAs contain regions which are primary precursors of specific tRNAs, it is quite likely that these regions would function poorly in normal tRNA reactions until they are processed by nucleases and/or tRNA modifying enzymes. Indeed the ability of TMV RNA to accept a methyl group from a specific tRNA methylase may be an indication of the absence of modified bases in the tRNA like structure. The isolation and structural analysis of this region of these viral RNAs would yield much information on the mechanism of tRNA processing and on the necessary requirements for efficient tRNA function.

Finally, the use of specific tRNA methylases as discussed in this report may be an excellent method for the detection of tRNA like sequences within any high molecular weight species of RNA as opposed to the use of aminoacyl tRNA synthetases which require a free 3' OH for esterification. rT forming uracil methylase is especially suitable as a "tRNA probe" since the modified base produced, rT, is characteristically found only in tRNA and has not been found in other types of RNA.

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